INDUSTRIAL MICROBIOLOGY

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PREFACE

For hundreds or even thousands of years mankind has practiced domestic arts in which microbes are the invisible but active agencies of desired change. Wine making, vinegar production, brewing, and the making of leavened bread were processes known to ancient peoples. Even when some of these processes became established on a larger scale the success was still somewhat dependent on chance and the combination of fortuitous circumstances with a degree of skill born of experience. Not until less than a hundred years ago was there any scientific appreciation of the real part microbes play in the transformations of organic matter.

Industrial microbiology is one of the important outgrowths of those fundamental researches conducted by Pasteur which have made his name the most highly revered in the whole realm of microbiology. Although his preeminence has been especially recognized in another field of microbic investigation which has greatly promoted man's welfare, the bacteriology of infectious disease, it should not be forgotten that his first studies were in fermentation and were conducted in aid of industries. He may thus especially be regarded as the founder of industrial microbiology as well as of medical bacteriology and immunology. Although the development of industrial microbiology was slow during the quarter century following Pasteur's work, a few outstanding contributions, such as those of E. C. Hansen, Jorgensen, Lafar, Delbruck, Duclaux, Lindner, and others, added materially to the subject before the opening of the present century. Since that time, development has been much more rapid, and industrial microbiology has now become a large and widely recognized field of study and practical application. In 1896 the senior author organized in the Biological Department at the Massachusetts Institute of Technology the first course of classroom instruction in industrial biology given in America. Of relatively small scope at the beginning, the course has been expanded from time to time as the subject has grown in importance, and the present related and parallel courses in industrial microbiology and food technology are outgrowths of its development.

The authors have prepared the present volume, believing that a comprehensive work in English dealing with this subject and presenting both theoretical and practical aspects of fermentation would be useful. Whether used primarily as a text for fairly advanced students or as a work

of reference, it is assumed in advance that the student or reader must already have had somewhat extensive training in biology and general bacteriology, and at least the fundamentals of organic chemistry and biochemistry. For this reason the general description and taxonomic treatment of the great class of the bacteria have been limited to the groups intimately concerned with the processes to be discussed later.

It is the purpose of this volume to outline, in a concise but comprehensive manner for students thus prepared, the fundamentals of industrial microbiology, and to present descriptions of the more important processes within the field.

No single text of moderate size can give a complete and detailed treatment of every aspect of the subject. There are in this volume what may, in some quarters, be regarded as serious omissions. For example, discussion of the commercial manufacture of sera, vaccines, and other therapeutic agents is omitted, as is also reference to the production of cultures of nitrogen-fixing bacteria, and of butter- and cream-ripening organisms, etc., although in a limited way each of these might be regarded as falling within the scope of industrial bacteriology. The reasons for these omissions will, it is believed, be evident on further thought—the first subject is closely related to public health and medicine; and the second subject seems more properly to belong to agricultural or to dairy bacteriology.

The efforts of the authors have been especially directed (1) to a broad treatment of those large-scale fermentations in which the end products are of themselves industrially or potentially important, and (2) to consideration of others that are of special significance because of the modifications of the quality of substrate materials that they produce, as in some aspects of food technology.

The various alcoholic fermentations as applied in brewing, wine making, and distillation industries; the acetic fermentation; the lactic fermentation; and the fermentations yielding butyl alcohol, acetone, citric acid, glycerol, gluconic acid, etc., are described and discussed since they represent type processes that illustrate basic principles and may supply the key to modes of inquiry that may be useful to the student in later researches or in the pursuit of new aspects of industrial fermentations.

A more comprehensive treatment of the general aspects of the subject will be found in the introductory chapter.

Consideration is also given to the discussion of some of the more recently discovered fermentations which have been investigated and which seem to offer possibilities of industrial significance in the near future. These are treated not only from the standpoint of the organisms involved but also with reference to the raw materials and production

methods that may be employed and to the biochemistry of the reactions catalyzed by the microbes.

The work has attempted to present the subject in a unified way, although it divides somewhat naturally into four parts, the first three dealing, respectively, with the biochemical activities of yeasts, of bacteria, and of molds. Within each of these three classes of microorganisms wth their extremely numerous and varied groups and species are found relatively few types having marked activity as causative agents of fermentation, but these are important since they represent types which probably can be employed economically and in large-scale operations. The last part of the book deals with specialized activities of microorganisms and some higher types of organisms in relation to undesirable changes produced in textile fibers, in wood itself, and in structures built of wood. Two appendices treat briefly of subjects useful to the industrial microbiologist. The first deals with the control of microbes by the use of germicides, and the principles of testing disinfectants, and the second with the treatment and disposal of industrial microbiological wastes.

At the end of each chapter, references are given, in order that the student who desires may continue his study of the subject by consulting original papers. The subject has now become so voluminous that it is impossible to present complete bibliographies, and although the references presented are regarded as the most useful for the student, it may be that numerous important publications have not been mentioned here.

The authors wish most gratefully to acknowledge their indebtedness to many who have assisted in the preparation of this book by permission to use tables, plates, and other illustrative material, and to others who have aided by the careful reading of some of the chapters. Special thanks are due Prof. W. H. Peterson and his associates at the University of Wisconsin; to Prof. C. H. Werkman and his colleagues at Iowa State College; to Drs. H. S. Knight, O. E. May, H. T. Herrick, A. J. Moyer, L. B. Lockwood, and their associates in the U.S. Department of Agriculture; to Dr. W. V. Cruess of the University of California; and to the editors of various journals, especially Dr. L. V. Burton and H. E. Howe, who have kindly given permission to use tables and figures. The authors are most appreciative of the kindness of Drs. G. B. Sippel and W. C. Tobie for their careful reading of the chapter on Brewing and the section on Rum Manufacture, respectively. Others to whom the authors wish to express gratitude are Dr. J. W. Lawrie of the Jos. Schlitz Brewing Company, Dr. C. N. Frey of the Fleischmann Laboratories, Dr. G. O. Lines of the Commercial Solvents Corporation, Dr. R. Schwarz of Schwarz Laboratories, and to their European fellow workers, Dr. A. J. Kluyver, Dr. A. Guilliermond, Dr. Ö. Winge, Dr. O. Meyerhof, Dr. H. Raistrick, Dr. C. G. Anderson, and Dr. Marjory Stephenson, for their friendly

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INDUSTRIAL MICROBIOLOGY

CHAPTER I

INTRODUCTION

Industrial microbiology is that portion of microbiological science which deals with the possible utilization of microorganisms in industrial processes, or in processes in which their activities may become of industrial or technical significance. Obviously the term "microbiology" in its broadest sense comprises that division of biological science which treats of the extremely small organisms of both plant and animal natures that Sëdillot in 1878 grouped together under the inclusive term "microbes." Although this word is now often used synonymously with bacteria it is not so limited, since within its field may actually be included all types of very small living things, such as the protozoa, spirochetes, and minute parasitic worms, as well as those low types of fungi commonly spoken of as the "yeasts" and "yeast-like organisms," the somewhat indefinite group called "molds," and the extremely numerous and widespread group of the bacteria. The modern general use of the term "protozoology," which has resulted from the many careful studies of the lowest group of animal organisms, has to a great extent in recent years transferred the interest and extended descriptions of many of the types of the animal group of *Protozoa* from the general field of microbiology to this more specialized domain of study. A modern tendency apparently is to confine the term "microbiology" largely to organisms that fall within the realm of the lowest groups of botanical or plant life. It is in this sense that the word is used in this book.

For several decades it has been known that numerous kinds of yeasts, molds and other low fungi, and several types or groups of bacteria have direct relation, either favorable or unfavorable, to certain types of economic processes carried out in connection with industrial or factory operations such as brewing, wine making, cheese making, etc., which have grown up from small-scale or family arts. Knowledge in this field has greatly extended in recent years, as research in many countries and in many lines has been carried out. The scale of operations has greatly enlarged and concentrated in manufacturing plants. Industries have replaced household arts. These changes have made it evident that

industrial microbiology is not only an exceedingly interesting branch of study but that it has already become a distinctly important branch of applied science, and one with even greater potentialities.

In any broad-gauge discussion of industrial microbiology it should be emphasized at the outset that knowledge in this field is of usefulness and significance from two diametrically opposite angles of application. On the first and more positive side, it is clearly advantageous to know the biological and biochemical characteristics of the many types of organisms that are the prime and direct causes of chemical transformation of materials into desired products. Here one utilizes the microorganisms capable of producing, by fermentation processes, relatively large quantities of chemical substances of usefulness and economic value. In this type of process the biochemical methods are in some instances the only economic methods of production. The manufacture of industrial alcohol and other industries based on alcoholic fermentation may be taken as a type of this class. Of equal interest to the microbiologist is the part played by organisms of these groups in composite operations, where the action sought is the production of somewhat smaller quantities of desirable by-products that should occur only as minor but important components in the final product. This is the aim in some branches of food manufacturing and in other commodity products.

On the other hand, it is recognized that fermentations incited by microbes may not always be desirable but may be quite the reverse. Competition by invading contaminants may wreck what would otherwise be a successful process. It is, then, of much importance to have knowledge of the organisms specifically useful in a process, and to be equally informed regarding those which oftentimes are destructive to or detrimental in manufacturing processes and are the causes of economic loss. The trained industrial microbiologist must be able to discover them, to recognize the type of damage they produce, and to become versed in the methods of combating them.

With all these viewpoints in mind, it is clear that industrial microbiology should include in its scope the study (I) of the numerous fermentation processes in which the production of alcohols, organic acids, glycerol, acetone, and other substances are end products; (II) of certain aspects of food-manufacturing processes, such as baking and the making of cheeses, butter, sauerkraut, and pickles, in which microbic agencies take a significant and important accessory part; (III) of food-conservation methods, such as canning and preserving, refrigeration, quick freezing, and drying, where sterilization or inhibition is imperative; and (IV) of the microbiological problems concerned with textile and commercial fibers. Obviously in the processes grouped under (II) and (III) and in a part of those under (I) industrial microbiology is intimately associated with food

technology. The work of the industrial microbiologist may also deal with the production and technical uses of bacterial and fungus enzymes, such as amylases and proteases, or with the preservation or protection of wood and the processes of commercial disinfection, wherein the application of suitable chemicals to restrain or prevent fungus, bacterial, or enzyme activity on walls and floors or on materials undergoing processing in the mill or manufacturing plant may make the difference between success and failure of operation.

The production of sera, vaccines, and other therapeutic agents commercially, although generally regarded as in the field of public health or medicine, and hence not here treated, might in reality be considered as a group of processes in industrial biology. It has seemed best to omit these processes in this volume, and also not to include the study of soil microbiology, the use of nitrogen-fixing bacteria, and the study of the phytopathology of economic crop plants, use of disinfectant or inhibitive sprays, etc. Although all these aspects of microbiology are extremely important in agriculture and therefore constitute a part of economic microbiology, they do not fall within the limits of this work.

In the present volume, industrial microbiology will be regarded essentially as the science and art of investigating and controlling technical fermentations, that is, of using microorganisms as reagents to produce desirable end products having possible or well-defined industrial uses and applications. Obviously, conditions in which losses due to contaminations or wholesale microbic infection affect manufacturing operations must enter into the subject matter. Although all the minute details of particular industries cannot be dealt with in a text of this scope, an attempt is made to present the principles and the general methods of operation. In addition to the consideration of the organisms and biochemical reactions concerned in the better-known industrial fermentations now in use, a few fermentations of present theoretical interest but perhaps potentially capable of technical development are also discussed.

It is important not to make too broad assumptions regarding industrial uses of microbes and to keep in mind the special qualifications which must be possessed by organisms in order that they may be economically utilized in the direct industrial production of materials having distinct commercial value. Obviously the number of species having this valuable property must be limited. The industrially important microbes may be characterized as having at least three outstanding qualities:

1. The ability to grow rapidly in suitable organic substrates and to be easily cultivated in large quantity.

2. The ability to maintain physiological constancy under these conditions and to produce the necessary enzymes readily and profusely, in order to bring about the desired chemical changes.

3. The ability to carry out these transformations under comparatively simple and workable modifications of environmental conditions; and, since the reactions are exothermic, without the application of large quantities of external energy.

Obviously, industrial operations with microbes are more complicated than laboratory experiments but involve the same principles. Large-scale operations must include protection from contamination, and the chemical engineering equipments must be carefully designed to meet the special conditions required in each type of fermentation.

Hundreds of microbes can grow rapidly in solutions of organic substances without yielding significant quantities of valuable products. They may bring about decompositions that conform to the broad general definitions of a fermentation that will be set up, but they may not be productive of a technically important or industrial fermentation.

Microorganisms of Fermentation.—As has been intimated, the microorganisms of fermentation include yeasts, molds, and bacteria. These microorganisms are unable to manufacture their own food by the ordinary process of photosynthesis since they lack chlorophyll and are classified as fungi, belonging to the phylum *Thallophyta.* Certain of the *Ascomycetes* (sac-fungi) and of the *Phycomycetes* (alga-like fungi), and a large number of species of the bacteria are the principal microorganisms that are directly concerned. A few of the *Basidiomycetes* are of significance in special types of breakdown of wood and fibers and are thus productive of changes comparable to fermentations.

The microorganisms of fermentation differ widely in respect to morphology, size, reaction to free oxygen, manners of reproduction, growth requirements, ability to assimilate or ferment raw (natural) substances, and in other ways. But they are similar in that they are "colorless" and grow most actively in darkness or diffused light, and all produce enzymes by which they catalyze the reactions ascribed to them.

Variation in Strains.—Even in a so-called "species" there may be a large number of types or strains, and, even in a so-called "pure strain," variation may occur under different environmental and nutritional conditions. Although the usual mode of increase by asexual reproduction favors the constancy of a given species, many factors may tend to cause changes in the chemical composition of a microorganism, the type and quantity of end products formed, and the rate of growth and reproduction. For example, the nature and quantity of the nutrient substances supplied, the temperature of incubation, the reaction of the medium, the oxygen relationships, the presence or absence of stimulating or inhibiting substances in the medium, and various other factors must be controlled and made similar in order to obtain analogous results with the same or related strains of a microorganism. The importance of mere traces of a sub-

stance cannot be too much stressed in some instances. Some of the apparent differences in results obtained in research in different laboratories with the supposedly same strain of organism may disappear when conditions of culture and the chemical composition of the media become exactly or essentially the same. It is, however, probably impossible to maintain perfect lack of variation over long periods.

Although some differences are only apparent ones, frequently true biologic variations exist in strains. Too much work has been carried out by responsible laboratories to leave any doubt as to this fact. For example, some strains of a microorganism apparently require added growth-accessory substances, while other strains require none; some strains of *Aspergillus niger* are stimulated by iron and zinc salts, others receive no apparent stimulation from these salts.

Fermentation.—From the biochemical standpoint, fermentation is the name given to the general class of chemical changes or decompositions produced in organic substrates through the activity of living microorganisms. Thus there may be many kinds of fermentation falling within this category depending on the type of organism involved, the type of substrate, or even the conditions imposed, such as pH, or oxygen supply. The word "fermentation" is a term that has undergone numerous changes in meaning during the past hundred years. According to the derivation of the term, it signifies merely a gentle bubbling or "boiling" condition, and the term was first applied when the only known reaction of this kind was in the production of wine. Even then no knowledge existed as to the cause. Thus in an active ethyl alcohol fermentation, as in a wine or cider fermentation, carbon dioxide is always liberated in the form of bubbles of gas, which at the height of the reaction may cause a marked agitation or movement of the liquid medium, especially in a large vat or tank, sufficient to give to it the appearance of a boiling liquid. This interpretation of the word was the accepted one for several thousand years. After Gay-Lussac studied the process the meaning was changed to signify the breakdown of sugar into alcohol and carbon dioxide. With the increase in knowledge following Pasteur's researches as to the *cause* of this change in the nature of the material fermenting, the word became associated with microorganisms and still later with enzymes, which are the biologically produced reagents by which microbes work. For a long time fermentation was especially associated with carbohydrates and, indeed, is often so considered at present, but a broader conception of these biologic reactions seems to be more logical. Thus putrefaction and the breakdown of fats by microbes are to be regarded as special kinds of fermentations.

Although fermentation is frequently or even generally associated with the evolution of gas due to the action of living cells, neither gas evolution

nor the visible presence of living cells is today regarded as an essential criterion of fermentation. In certain fermentations, for example, some of the lactic fermentations, no gas is liberated. Again, fermentation might result (although it is not frequently the case) from the use of cell-free enzyme extracts that for a time catalyze the reactions. Gas can be liberated in some of these processes, as for example with a zymase preparation. Cell-free fermentations are, however, unknown in nature and when produced in the laboratory are considerably slower than fermentations where the living cells are present, and are largely of theoretical interest.

Fermentations are so varied in character that any general definition that will cover this whole class of biochemical processes must be couched in very general terms. Nevertheless, it is sometimes useful to gain a briefly stated concept of such a group of reactions. The idea might be expressed as follows:

A fermentation, in the broad sense in which the term is now used, may be defined as a process in which chemical changes are brought about in an organic substrate, whether carbohydrate or protein or fat or some other type of organic material, through the action of the biochemical catalysts known as "enzymes," elaborated by specific types of living microorganisms.

For more exact characterization, the kind of fermentation, such as alcoholic, lactic, acetic, etc., must be specified. Since the majority of fermentation processes first studied concerned carbohydrates, these are often regarded as the essential materials, but it must be clear that proteins, fats, some salts of organic acids, and alcohols can be broken down by similar agencies and thus fall within the classification of fermentable material.

Enzymes.—The student of biology or biochemistry will already have learned of the universal association of enzymes with living matter and the important part played by them in all types of vital activity. The activities of enzymes are especially prominent in the phenomena of digestion in animals, in the transformations of starch in sprouting seeds, etc. These and many other observations lead to the view that enzymes are the essential organic catalysts, possessed or produced by all living cells, without which the processes of life would cease or be impossible. It is, furthermore, well established that these enzymes are of two types, called "exoenzymes" and "endoenzymes," according to their sphere of activity outside or within the confines of the cells that elaborate them. Both types are extremely important. Exoenzymes, liberated by the manufacturing cells, penetrate and break down the organic materials outside the cell, such as the proteins, starches, and fats of food materials, giving rise to soluble derivatives and so making it possible for the prod

of their activity to be absorbed through the cell membrane. Energy liberated by the enzyme action outside the cell is of comparatively little value to the cell. In general it is found that exoenzymes liberate relatively little energy, as heat, especially in the most commonly produced hydrolytic processes.

The endoenzymes elaborated and retained within the living cell, on the other hand, behave quite differently. The food substances, having been absorbed into the cell, may be further transformed and broken down by the action of the endoenzymes, and this process is accompanied by the liberation of relatively large amounts of energy, this energy being available to the cell.

The microorganisms of fermentation are notable for their ability to produce enzymes of both these classes. Molds, yeasts, and bacteria can secrete or elaborate a wide variety of enzymes, possibly of a greater range than any other single cells, since all the functions of growth, reproduction, digestion, assimilation, etc., that are distributed among the various tissues and organs of higher plants and animals seem here to be concentrated in the single minute cell. This may explain why it is that, in these low groups of extremely small plants which are essentially unicellular in structure, we find a higher degree of enzyme productivity and of fermentative capacity than is exhibited elsewhere in the world of living things.

The enzymes are therefore the reactive substances or catalyzers that microorganisms employ in bringing about the specific changes or fermentations that are characteristic of different species or groups of microbes. One can visualize each strain, species, or genus as having its own armamentarium of enzymes or its own peculiar ability to secrete them. In this individual potentiality may lie one of the factors that account for the variations in strains mentioned earlier in this chapter.

Energy Relationships.—The following table, which shows the energy liberated from 1 g. of substrate by enzymes, will give some insight into the relative energy values of exoenzymes and endoenzymes.

TABLE 1.—ENERGY LIBERATED FROM ONE GRAM OF SUBSTRATE BY ENZYMES[1]

Exoenzymes	Calories	Endoenzymes	Calories
Pepsin....	0	Lactacidase.......	82
Trypsin...	0	Alcoholase........	149.5
Rennet...	0	Urease...........	239
Lipase....	4	Oxidase (vinegar).	2,530
Invertase.	9.3		
Maltase..	10		
Lactase...	23		

N, C. G., "An Introduction to Bacteriological Chemistry," Williams, Wood & Company, The Williams & Wilkins Company, Baltimore, 1938.

The question naturally arises, does the microorganism have a requirement for a major portion of the large amounts of energy liberated within the cell, or is much of the energy liberated merely due to the great excess of enzyme product or the result of unrestricted enzyme action? Experiments with yeast cells indicate rather emphatically that the energy evolved is not a measure of metabolic requirements, but is the result of enzymes acting on an abundant and suitable substrate. However, further experimentation is necessary to lend additional weight to this suggestion. Whatever the theoretical aspects, it is known in practice that under the artificial conditions of a great volume of fermenting liquid, in which billions of cells are active, energy in the form of heat may be liberated to a marked degree. In such large masses of fermenting material where the heat produced cannot be readily diffused or conducted away the rise of temperature may become so great as to impair the organisms that produce the reacting catalyst. In vinegar fermentation the heat energy evolved may be sufficient to cause a rise of temperature that may inhibit normal cell growth and stop further activity.

Intense Activity of Microorganisms.—Many microorganisms, in comparison to the higher organisms, are intensely active in respect to the chemical changes that they bring about. The examples cited above show this fact and also are typical of the fact that all fermentative processes are exothermic. Burchard[1] has calculated that 1 g. (wet weight) of *Micrococcus ureae* decomposes 180 to 1,200 g. of urea per hour, while Haacke estimated that 1 g. (wet weight) of a lactose fermenting organism breaks down 178 to 14,890 g. of lactose in 1 hr. Even though these calculations may be of only approximate value, the enormous activity of microorganisms is evident. This great chemical activity of microorganisms is associated with their simple life requirements, the ease with which they attack food for energy, the rapidity of growth or reproduction, and possibly their capacity for maintenance under different conditions.

Specific Types of Fermentative Change.—It has been intimated in the foregoing pages that the specific or characteristic kind of fermentation produced by an organism is dependent on its enzyme-producing powers. One might expect, therefore, that those organisms most nearly related generically would be most similar in their fermentation relationships. This seems to be generally the case, although it does not follow that organisms belonging to different biological groups are necessarily totally unlike in their ability to catalyze fermentation processes having some of the same end products. The "true" yeasts and some other budding fungi nearly related to them are the organisms that most commonly produce ethyl alcohol, and because of this fact it is often stated that the

[1] Stephenson, M., "Bacterial Metabolism," Longmans, Green & Company, 1930.

yeasts are the microorganisms of alcoholic fermentation, but there are many budding fungi, classified in groups or families morphologically very similar to yeasts, that apparently have no ability to produce ethyl alcoholic fermentation or any other with large quantities of a particular end product. On the other hand there are a few fungi, somewhat higher in the scale of organization than yeasts, that can produce ethyl alcohol under certain conditions of substrate, pH, relation to oxygen, etc. Even a very small number of species of bacteria can produce alcohol. These are unusual cases but apparently demonstrate the great versatility of the low colorless plants in developing their enzyme systems. In general, the molds that have marked fermentation power are producers of organic acids and of products of protein decomposition. Similarly, the fermenting types of bacteria commonly give rise to organic acids, frequently different from the acids produced by molds, and to higher alcohols than ethyl. It cannot be assumed that all species of microorganisms belonging to the three groups that have been mentioned as constituting the microorganisms of fermentation actually have marked ability in this respect. It would be more nearly correct to say that the significant fermentation organisms represent a minority of all the organisms so classed.

Energy Value of Substances.—The substances acted on by microorganisms present a dual role, the first as a food, the second as a material to be transformed through the action of enzymes that have been produced in excess of nutritional requirements. In general, the energy value of a substance, such as glucose, depends on the degree of oxidizability it can undergo. In the complete oxidation of glucose, as in burning, considerable energy is evolved. This is also true in the breakdown by aerobic organisms, while in the anaerobic breakdown of this sugar only a fraction of the potential energy is liberated. Consequently, in order to obtain an equivalent amount of energy, several times as much glucose must be broken down under anaerobic conditions as would be required under aerobic conditions.

The breakdown of nutrients in fermentation is not merely expressed in the evolution of heat but is in early stages at least always accompanied by division of new cells. For example, in the manufacture of compressed yeast, the nutrient medium is supplied not only with organic food but also with a very large amount of air. Aerobic oxidations are favored and under such conditions, along with the fulfillment of nitrogen and phosphate requirements, pH control, and the employment of a low concentration of sugar, large yields of yeast cells are produced. Under essentially anaerobic conditions, such as would exist in the normal industrial fermentation for ethyl alcohol, much more sugar must be utilized to produce the same quantity of yeast cells, and a larger amount of the organic matter of the substrate is converted to the characteristic end

products, one of which, ethyl alcohol, is still comparatively high in potential energy.

It should now be clear that a fermentation process that may become of industrial significance is not a simple matter of inoculating organic matter with microbes that can derive food from it, but is instead the setting up of a system of biochemical reactions between a substrate and an organism that can partially decompose it in a special manner under controlled and workable conditions. The substrate must be abundant, inexpensive, and fairly high in potential energy. The organism must have ability to attack, by means of its enzymes, certain chemical groupings in the composition of the substrate and, by hydrolysis, oxidation or reduction, or other means, disrupt certain bonds in the substrate molecules and give rise to stable products under the imposed conditions. Thus by a single reaction or a series of coincident or successive enzyme reactions carried out under definite conditions it may bring about eventually a desired product or group of products of lower potential energy.

CHAPTER II

THE YEASTS

The yeasts and other yeast-like organisms, often grouped together under the name *Blastomycetes* or "budding fungi," belong to the subdivision of the thallophytes designated as the *Eumycetes*, or true fungi, since they possess no chlorophyll. All the organisms here grouped are unicellular plants of microscopic size and widely distributed in nature, and they occur especially in the top layers of the soil, in dust, and on the fruits and leaves of many plants. The grape, apple, pear, and many other fruits have these organisms almost constantly present, and the soil of orchards and vineyards where the microbes live over the winter is particularly well populated with yeast cells. Distribution is easily effected by wind and on the bodies of bees, wasps, and other insects.

The budding fungi may be separated into two rather unequal divisions: the spore-forming (sporogenous) or true yeasts represented by the family *Endomycetaceae* (*Saccharomycetaceae*); and the nonsporing (asporogenous) pseudo or false yeasts, which are represented by the families *Rhodotorulaceae*, *Torulopsidaceae*, and *Nectaromycetaceae*. The true yeasts include about 17 genera and a large number of so-called "species," many of which in turn show numerous slightly different strains or "types." Although most taxonomists are in agreement as to the main groups, definite classification into genera and species is extremely difficult and unsatisfactory from the botanical standpoint. Industrially, however, the one genus *Saccharomyces* is of outstanding interest, as most yeasts having any technical uses belong herein.

The yeasts grow most luxuriantly in solutions containing sugar and the other necessary food requirements. In a fluid nutrient medium the cells ordinarily occur singly or in twos or threes or attached to one another in small clusters that are actually groups of cells of different generations, as a result of the characteristic method of vegetative increase by budding. On agitation, these groups break apart, and most of the cells eventually settle to the bottom of the container as sedimentary cells. If the culture medium remains undisturbed, islands of cells or films of greater or less complexity may appear on the surface. Sometimes these superficial cells may appear as chains or filaments of cells, resembling hyphae; and often with a number of smaller cells budded off at the nodes. Some true yeasts, such as those of the genus *Endomyces*,

may form a true mycelium or mass of cells. On favorable solid media, compact colonies are produced, varying in size and surface markings according to the nature of the substrate and the age of the colony.

Shapes of Cells.—The individual yeast cells are usually spherical, ovoid, or ellipsoid in form. Broadly egg-shaped and elongated sausage-shaped cells may be produced characteristically by certain yeasts, nevertheless, the shape of the active cell is not an exact means of species

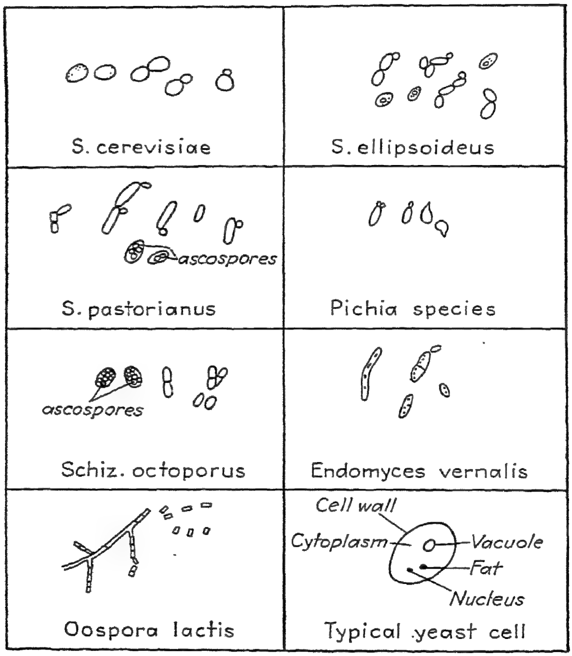

FIG. 1.—Some different types of yeast cells (*Oöspora lactis*, excepted).

identification, nor is variety in form in the same culture a proof of contamination. Yeasts possess no flagella, and consequently the individual cells are nonmotile. The accompanying figure illustrates a few of the different types of yeast that occur in nature.

Size.—Yeast cells may vary considerably in dimensions, depending on the species, nutrition, age, and other factors. The cell may vary from 1 to 5 or more microns in width and from 1 to 10 or more microns in length. With the approximately spherical cells of industrial yeasts a diameter of 4 to 6 microns is probably a fair average, but great variations

may occur even in the same culture. Most of the yeasts of extremely small size are of no present industrial importance except as they occur as contaminants.

In general, yeast cells are much larger than bacterial cells and could not be mistaken for the latter when observed microscopically. There are exceptions, however; some large bacteria exceed in size the smallest known yeasts mentioned in the figures given.

The Cell Wall.—A transparent permeable wall surrounds each cell or mass of protoplasmic material. The exact composition of the cell wall is not known. It is believed to be composed of "fungus cellulose," which differs in its chemical character from the cellulose complexes constituting the walls of green plants. The cell-wall membrane may be invisible or very thin in young cells but becomes thickened in old cells.

Contents of the Cell.—The protoplasm appears microscopically as composed of a grayish, finely granular, semifluid mass. Presumably it is of albuminous material intimately mixed with a "cell sap" of water with organic materials and salts in solution. Within the protoplast there is a nucleus and one or more rounded structures known as "vacuoles," which were originally so called because they appeared as clear or empty spaces but are now known to be the seat of reserve foods and finely divided "metachromatic granules" or volutin. The vacuoles are not conspicuous in very young cells but appear prominently in mature or old cells, and their nature can be somewhat determined by special staining methods. Generally a large vacuole is located near the nucleus, or there may be two or even more with a general polar location. The nucleus exists as a small mass near the center of the cell and cannot generally be seen without employing a special staining procedure. Iron hematoxylin is probably the best stain for this purpose. With this treatment the nucleus sometimes appears as a fairly compact body, but more often as a diffuse cluster of granules. The nucleus assumes an important role in reproduction. When budding takes place the nucleus moves toward the pole, and a portion of these granules (possibly half of them) migrates into the newly developing daughter cell.

Within the yeast cell protoplasm are numerous other substances or reserve materials in the form of droplets or granules, some of which are exceedingly complex in nature. In old cultures, some cells sometimes become thick walled and much enlarged and densely packed with these reserves. Such cells are sometimes called "durable" or "durative" cells, as they seem to have unusual resistance to adverse conditions.

These stored foods—including some carbohydrates (not starch), fats, or oils as refractile droplets, and some granules supposed to be proteins—are normal constituents of all mature yeast cells but especially of the older cells, when nutritive conditions are favorable. As stated, this reserve

may form a true mycelium or mass of cells. On favorable solid media, compact colonies are produced, varying in size and surface markings according to the nature of the substrate and the age of the colony.

Shapes of Cells.—The individual yeast cells are usually spherical, ovoid, or ellipsoid in form. Broadly egg-shaped and elongated sausage-shaped cells may be produced characteristically by certain yeasts, nevertheless, the shape of the active cell is not an exact means of species

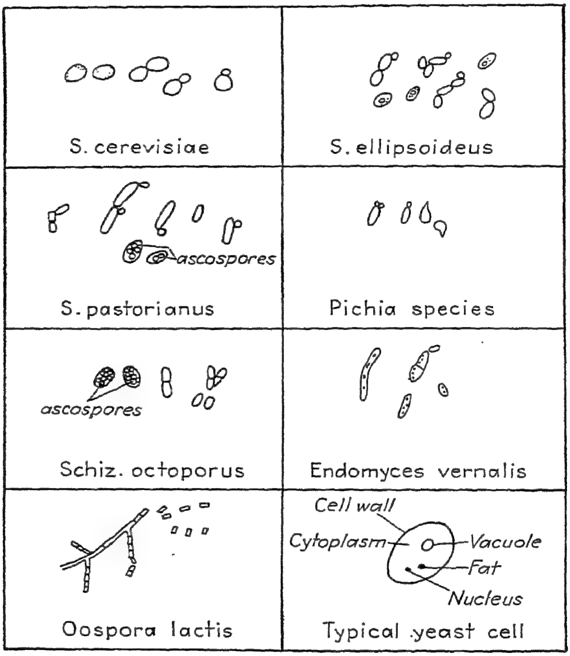

FIG. 1.—Some different types of yeast cells (*Oöspora lactis*, excepted).

identification, nor is variety in form in the same culture a proof of contamination. Yeasts possess no flagella, and consequently the individual cells are nonmotile. The accompanying figure illustrates a few of the different types of yeast that occur in nature.

Size.—Yeast cells may vary considerably in dimensions, depending on the species, nutrition, age, and other factors. The cell may vary from 1 to 5 or more microns in width and from 1 to 10 or more microns in length. With the approximately spherical cells of industrial yeasts a diameter of 4 to 6 microns is probably a fair average, but great variations

may occur even in the same culture. Most of the yeasts of extremely small size are of no present industrial importance except as they occur as contaminants.

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These stored foods—including some carbohydrates (not starch), fats, or oils as refractile droplets, and some granules supposed to be proteins—are normal constituents of all mature yeast cells but especially of the older cells, when nutritive conditions are favorable. As stated, this reserve

food is found dissolved in the cell sap in vacuoles and in the form of granules or droplets.

The most important carbohydrate is glycogen $(C_6H_{10}O_5)_x$, but other polysaccharides have been detected. Trehalose, a disaccharide $(C_{12}H_{22}O_{11})$ yielding glucose when hydrolized by acids, phosphatides, enzymes, vitamins, growth substances, volutin, and, in some instances, pigments are found in yeasts.

Some yeasts store relatively large quantities of fat (see the section on fat production). One yeast, *Endomyces vernalis,* produces so much fat under suitable conditions that it was used as a source of fat for human consumption during the World War. Fat collects in vacuoles that often become highly refractile as the cell ages. One school of microbiologists regards the storage of fat as an indication of cell degradation.

Volutin, also referred to as metachromatic granules, occurs in vacuoles, of which is nearly always located close to the nucleus. It is a reserve terial and is believed to be nucleic acid or a nucleic acid compound. is found usually in old cells, but tends to disappear during spore formation.[1]

Stains.—A list of some of the stains used in detecting the presence of various chemical substances in the yeast cell follows:

TABLE 2.—SOME STAINS USED TO DETECT SPECIAL SUBSTANCES IN THE YEAST CELL

Stain	Substance	Color produced in substance
Flemming's solution[1]	Fat globules	Brown to black
Sudan III	Fat globules	Red
α- or β-Naphthol plus dimethyl-*p*-phenylenediamine in a weakly alkaline sol	Fat globules	Blue
Dimethyl amido-azobenzene	Fat globules	Yellow
Neutral red (0.1%)	Metachromatic particles, vacuoles	Light pink to red
Polychrome methylene blue.	Nucleoprotein of volutin	Blue
Zinc chloriodide	Cellulose	Blue
Iodine in potassium iodide	Glycogen	Red-brown
Iodine in potassium iodide	(Starch)	Blue-violet

[1] Flemming's solution: 15 parts of a 1 per cent aqueous solution of osmic acid, 1 part of crystallized acetic acid, 4 parts of a 2 per cent aqueous solution of chromic acid.

Chemical Composition.—Yeasts contain 68 to 83 per cent of moisture,[2] while bacteria contain 73.3 to 98.3 and molds 84.3 to 88.7 per cent.

[1] HENRICI, A. T., "Molds, Yeasts and Actinomycetes," John Wiley & Sons, Inc., New York, 1930.

[2] BUCHANAN, R. E., and E. I. FULMER, "Physiology and Biochemistry of Bacteria," Vol. I, Williams & Wilkins Company, Baltimore, 1928.

Protein, carbohydrate, fat, and ash contents vary, depending on the species of yeast and the conditions under which it was grown, the ash content of yeasts usually varying from 3.8 to 8.8 per cent on the basis of the dry matter. In the following table is an analysis of the dry matter, which constituted 30 per cent of the total weight of yeast.[1] It will be noticed that yeast is rich in protein and glycogen, also that phosphorus and potassium combinations make up about 91 per cent of the ash. Phosphates play a very important role in fermentation.

TABLE 3.—COMPOSITION OF THE DRY MATTER OF YEAST[1]

	Per Cent		Per Cent
		Ammonia	8
Protein	52.41	Purine and pyrimidine bases	12
		Diamino acids	20
		Monamino acids	60
Fat	1.72	Phosphorus pentoxide	54.5
Glycogen	30.25	Potassium oxide	36.5
Cellulose, gum, etc	6.88	Magnesium oxide	5.2
Ash	8.74	Calcium oxide	1.4
	100.00	Silicon oxide	1.2
		Sodium oxide	0.7
		Sulphur trioxide	0.5
		Chlorine and iron	Trace

[1] FREY, C. N., History and Development of the Modern Yeast Industry, *Ind. Eng. Chem.*, **22**: 1154 (1930).

The proteins of yeast include albumin, globulin, phosphoproteins, nucleoproteins, lecithoproteins and glycoproteins. Soluble protein derivatives—peptones, polypeptides, and amino acids—are normal constituents of the cell.

Reproduction.—The true yeasts, *Endomycetaceae*, may reproduce vegetatively by budding or by fission and also by spore formation.

During budding, cytoplasm and nuclear material flow from the mother cell into a growing bud or protruding portion of the cell (Fig. 2). The bud becomes constricted at its base, a wall forms across the channel, and the cell may break off at once or remain attached to the mother cell for a while. Not infrequently the mother cell may form buds in two or more places at once. Clusters of cells thus result when the new cells remain temporarily in connection with the parent cells.

Another vegetative type of reproduction is transverse fission. This process is similar to that of bacterial fission. The mother cell grows, elongates, becomes somewhat constricted in the middle, and a division wall is formed, two new cells resulting. The genera *Schizosaccharomyces* and *Endomyces* reproduce vegetatively only by transverse fission.

[1] FREY, C. N., History and Development of the Modern Yeast Industry, *Ind. Eng. Chem.*, **22**, 1154 (1930).

Sporulation, a reproductive process in true yeasts, may be of asexual or sexual nature. The spores, of which there may be 1 to 4, 6, 8, or very

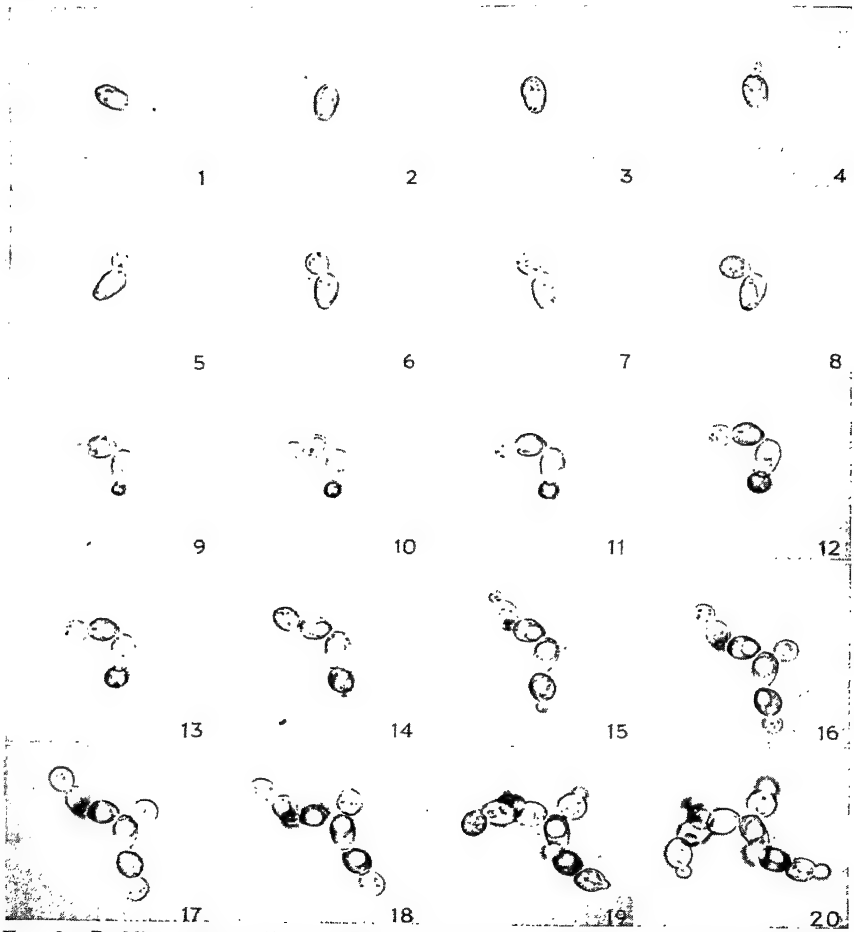


FIG. 2.—Budding yeast cells. Magnified 1,500 times. Pictures taken at 15-minute intervals. (*Courtesy of Dr. C. N. Frey of the Fleischmann Laboratories.*)

infrequently 12, are known as "ascospores" or "endospores," and the cells that contain the spores are known as "ascs" or "asci" (singular, "ascus").

True yeasts are classified as *Ascomycetes* (sac fungi).

In ordinary ascospore formation, the nucleus of the yeast cell divides repeatedly. Each nucleus is then surrounded by dense cytoplasmic material and finally a wall.

When the nuclei of two different yeast cells fuse prior to the division of the nucleus, the resultant spores are sexually produced in nature—this type of spore formation is much less common than the asexual method, *i.e.*, without fusion.

Conjugation may be isogamic or heterogamic. In isogamic conjugation the nuclei of two neighboring cells of about equal magnitude fuse. In heterogamic conjugation, the nucleus of the bud fuses with the nucleus of the mother cell, or nuclei of cells of very unequal dimensions fuse. The fusion nucleus divides and the ascospores form. The genera *Debaromyces* and *Zygosaccharomyces* illustrate isogamic and heterogamic conjugation.

Yeast Spores.—Sporulation in yeasts is important for two reasons: it is the basis for a method of reproduction, and it serves an important role in maintaining the viability of yeasts during adverse changes in the environment. The ascospores of yeasts are more resistant to heat and desiccation than the vegetative cells, yet they are much less resistant to heat than bacterial spores.

Sporulation.—Sporulation may be initiated by a deficiency in the food supply and the accumulation of toxic end products[1] but will not proceed unless certain other conditions are favorable, namely, the yeast cells must be young and vigorous; there must be plenty of air and moisture available; the pH of the medium must be suitable; inhibitory substances must be absent; and the temperature of incubation must be satisfactory. The presence of stimulating substances promotes sporulation.

In order to produce vigorous young cells, the yeast should be cultured in a suitable medium and transferred frequently. Reserve foods—glycogen, fat, and other products—are stored in the yeast. The use of such cells will insure sporulation, provided the other conditions are satisfactory and the yeast has the ability to form endospores.

Oxygen is essential for sporulation; without it no spores are formed.

Temperature is important. Each variety of yeast sporulates most readily in a given temperature range. Above certain temperatures and below others, sporulation fails to take place. Table 4 shows the maximum, minimum, and optimum temperatures for the sporulation of six varieties of yeasts studied by Hansen.

The optimum temperatures for the six varieties lie between 25 and 30°C., while three varieties have an optimum temperature of 25°C. At

[1] Guilliermond, A., "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

the most favorable temperature for sporulation, ascospores begin to appear in 21 hr. or more.

TABLE 4.—MAXIMUM, MINIMUM, AND OPTIMUM TEMPERATURES FOR SPORULATION OF CERTAIN YEASTS[1]

Yeast	Maximum temperature, degrees Centigrade	Minimum temperature, degrees Centigrade	Optimum temperature, degrees Centigrade
Saccharomyces cerevisiae	35 –37	9 –11	30
Saccharomyces pastorianus	29 –31.5	0.5– 4	27.5
Saccharomyces intermedius	27– 29	0.5– 4	25
Saccharomyces validus	27 –29	4.8– 5	25
Saccharomyces ellipsoideus	30.5–32.5	4.7– 5	25
Saccharomyces turbidans	33 –35	4 – 8	29

[1] Reprinted by permission from Guilliermond, "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

Calcium sulphate stimulates sporulation but restrains budding. Beer containing glucose or other sugars or gelatin stimulates sporulation, but ammonium salts have an adverse affect. Green, blue, violet, and ultraviolet rays have inhibitory effects on the formation of endospores. Extremes of pH definitely retard sporulation.

Methods of Inducing Sporulation. 1. *Plaster of Paris Block.*—In this method, introduced by Engel, the upper surface of a block molded from pure plaster of Paris is scraped smooth. The block is placed in a glass container, which is fitted with a cover, and water is added until the block is about one-half submerged. The container, cover, and contents are then sterilized. Yeast cells from a young, vigorous culture, which has been grown on a suitable medium with frequent transfer, are placed on the smooth surface of the block, and the container with contents is incubated at the desired temperature (25 to 30°C. usually) for 30 hr. or longer before observations are made. Hansen has devised a special flask for use in this method. Clay and blotting paper have been substituted for plaster of Paris.

2. *Gorodkowa Medium.*—Very satisfactory results have been obtained by inoculating slants containing Gorodkowa's medium with an active culture of yeast:

Glucose	0.25 g.
Beef extract	1.00 g.
Agar or gelatin	1.00 g.
Sodium chloride	0.50 g.
Distilled water	100.00 cc.

3. *Carrot Infusion Agar (McKelvey).*—An agar medium prepared from a weak infusion of carrots and containing a small amount of calcium sulphate gives good results. Slants of this medium are inoculated as above.

4. *Other Methods.*—Yeast will sporulate on many other media, provided that the nutrient substances are available in scanty portions. Yeast water, thin layers of liquids containing but little food, gelatin, and slices of carrot and potato have been used to encourage sporulation.

The failure of one method to produce spores does not necessarily indicate that the yeast concerned does not sporulate, for yeasts vary in their response to different media.

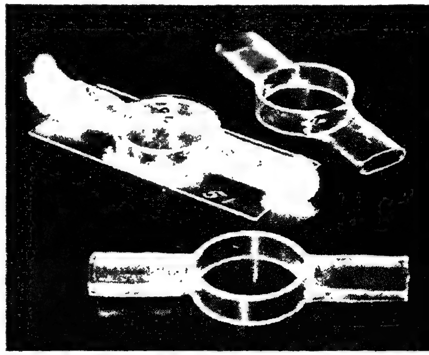


FIG. 3.—Chamber used in isolating single spores. (*Courtesy of Dr. Ö. Winge, Carlsberg Laboratory, Copenhagen.*)

The Isolation of a Single Spore.—Winge and Laustsen[1] regard the one-spore culture as being the smallest biological unit in yeasts instead of the single cell.

Laustsen has developed a technique for isolating all the spores of an ascus and cultivating them. His method is as follows:[1] The yeast is caused to sporulate by placing it on a plaster block and incubating it for at least 30 hr. at room temperature. Using aseptic technique, a small drop of wort on a cover slip is inoculated with the spore material. The cover slip is then placed on a special operating chamber (Fig. 3).

An ascus is selected and withdrawn from the droplet, using a very fine glass needle. A small amount of wort naturally adheres to the ascus —more may be readily added if desired.

The isolation (Fig. 4) is carried out by means of two special glass needles. One needle, which has a point about 7 microns in diameter, is introduced through one opening of the operating chamber (Fig. 3); the other needle, which has a point approximately 2.5 microns in diameter, is introduced through the other opening. The two needles are manipulated in such a manner as to burst the wall of the ascus against the surface of the cover slip. Each of the spores liberated is pulled into a separate droplet of sterile wort, which has been placed previously on the cover slip.

After the spore has germinated and a colony of cells has developed, some of the cells are transferred by the use of a sterile platinum needle

[1] WINGE, Ö., and O. LAUSTSEN, *Compt. rend. trav. lab. Carlsberg, Sér. Physiol.*, **22** (No. 6): 99 (1937).

to a Freudenreich flask containing sterile wort. Some of the remaining cells may be transferred to a second flask by using a sterile, pointed piece of filter paper.

Occurrence and Distribution of Yeasts.—The budding fungi are of wide distribution in nature. Their habitats may include not only the upper layers of the soil but also many forms of organic matter, especially of plant origin, where carbohydrates are of common occurrence. Yeasts may be isolated particularly from the soil of vineyards and orchards; from the surfaces of grapes, apples, and most sweet fruits; from citrus fruits;

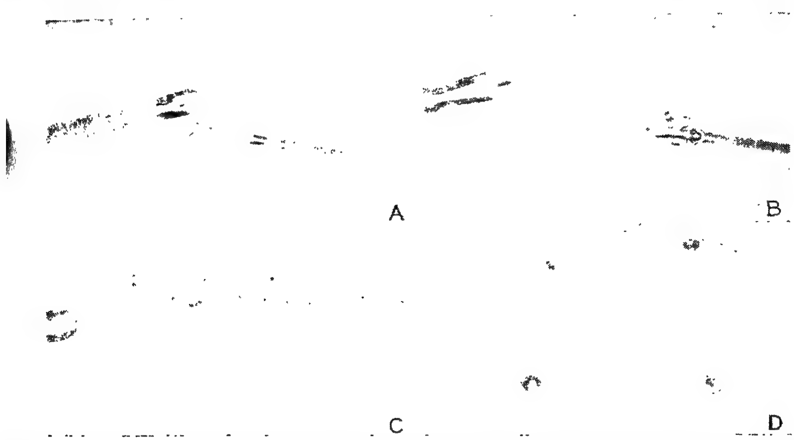


FIG. 4.—The isolation of single spores. (*Courtesy of Dr. Ö. Winge, Carlsberg Laboratory, Copenhagen.*)

and from the leaves and other parts of plants. They are carried into the air with dust and on the bodies of insects and thus may be widely disseminated. Proctor[1] has shown that yeasts may be found in the air at high altitudes. Some types of yeasts are also found occasionally on animal products.

Methods of Isolating Pure Cultures of Yeast.—In order to make sure that the culture isolated is pure and that it is not in reality a mixed culture of two morphologically related varieties of yeast or an undesirable combination of microorganisms, it is necessary to isolate single cells by special technique and to observe these cells during reproduction. Although there are several methods by which pure cultures may be isolated, the methods cited here will be restricted to the more important ones. Skill and patience are required in some of the methods of single-cell isolation.

[1] PROCTOR, B. E., *Proc. Am. Acad. Arts Sci.*, **69**: 315 (1934).

1. *Moist-chamber Method of Hansen.*[1]—The chamber (Fig. 5) consists of a glass slide, a glass ring, and a cover slip with numbered squares (usually 16). The cover slip is attached to the ring by glass cement, wax, or a vaseline-wax mixture. Before using the chamber, the component parts should be sterilized by flaming them carefully.

The culture of yeast from which the single-cell isolation is desired is diluted with sterile beer wort or water until a drop contains only a few cells. A drop of the diluted culture is then placed in a tube of sterile melted wort gelatin, and the tube is thoroughly agitated to distribute the yeast cells uniformly throughout the medium. One drop of this medium is spread out on a glass slide, and, using the 100 × magnification of a microscope, the slide is examined for the approximate number of cells.

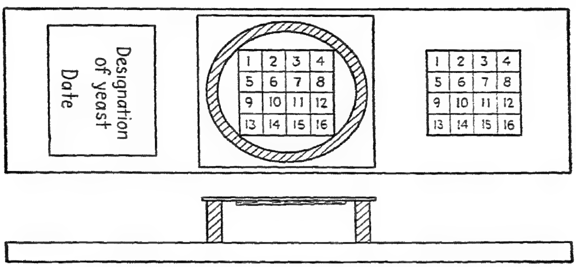


Fig. 5.—Moist chamber for Hansen method.

If the number is satisfactory (20 or less), a small drop of the liquid wort gelatin containing the cells is placed on the underside of the cover slip and spread thinly and uniformly over the numbered squares. The chamber is placed so that the gelatin rests in an even layer on the cover slip until it has solidified. Afterward the chamber is inverted, a drop of sterile water is placed on the slide to ensure a moist atmosphere in the chamber, and the ring is sealed tightly to the slide by means of vaseline. The single cells are then located microscopically, and their positions are mapped on a diagram corresponding to the marked cover slip. Several moist chambers are usually prepared at the same time. They are then incubated at room temperature or around 25°C. for 2 or more days, during which time the growing yeast cells are carefully observed. During this time the single cells will have developed into colonies. Tubes of sterile wort are then inoculated from the individual colonies known to have grown from single cells.

2. *The Lindner Method.*—This method is a modification of the Hansen procedure. The culture containing the desired yeast is diluted in sterile

[1] Jorgensen, A., "Practical Management of Pure Yeast," revised by A. Hansen, Charles Griffin & Company, Ltd., London, 1936.

wort, cider, or grape juice. Using a sterile crow-quill pen or wire, five rows of droplets, 5 droplets to a row, are deposited on a sterile cover slip that may or may not be marked into numbered squares. The cover slip is placed, culture side down, over the concavity of a sterile hollow ground-glass slide or ring slide after placing a drop of sterile water in the chamber. It is sealed with vaseline or paraffin. The droplets are examined microscopically, and those containing a single cell are marked by drawing small circles around them, or, if the squares are numbered, the locations are recorded by sketching the position of the droplet on a corresponding drawing. After incubation and observation, the cultures are transferred to sterile wort.

3. *Dilution-plate Method.*—Tubes of dextrose, malt, or other suitable agar are melted and cooled to 42 to 44°C. Using a loop, a tube of agar is inoculated with the yeast-containing culture. The tube is thoroughly shaken, a unit amount of this medium is transferred to a second tube of melted agar. A third tube is inoculated in the same manner from the second tube. The contents of each of the three tubes are poured into petri dishes, and the plates incubated at 25 to 30°C. for 2 or more days. When the colonies have developed, samples of those which appear to consist of pure yeasts are examined with the microscope, using hanging drops. If the yeast appears to be pure and satisfactory, sterile wort may be inoculated from the colony. This method, though simple, does not ensure the isolation of a culture from a single cell.

4. *The Micromanipulator Method.*—The micromanipulator may be used successfully in isolating pure cultures of yeasts from single cells. In experienced hands, this method is rapid. The euscope, attached to the microscope, aids in the isolation of single cells.

Giant Colonies.[1]—Lindner suggested that giant colonies may contribute information that would be useful in identifying a yeast. A large horizontal surface of a suitable nutrient agar or gelatin medium contained in an Erlenmeyer flask or a large bottle is inoculated at one point in the middle with the aid of an inoculating needle. Petri dishes may be used, but owing to their susceptibility to contamination, they must be wrapped with surgeon's tape or packed in sealed containers to prevent contamination. Incubation is usually made for a period of 1 to 2 months at or near 20°C. The use of moist chambers aids in preventing the agar from shrinking prematurely.

Identification of Yeasts.—Yeasts are identified on the basis of a large number of observations—morphological, physiological, biochemical, cultural, and others. The shape and size of the cell; the optimum,

[1] Guilliermond, A., "Clef dichotomique pour la détermination des levures," Librairie Le François, Paris, 1928; "The Yeasts," translated and revised by F. W. Tanner, *op. cit.*

minimum, and maximum temperatures for budding, sporulation, and film formation; the copulation of cells; the morphological nature of the ascs and ascospores; the method in which ascospores germinate; the characteristics of the sediment formed in wort; the appearance of colonies grown on various solid media; the characteristics of giant colonies; and the biochemical characteristics, such as the action on various sugars, are some of the more important factors considered in identifying an unknown yeast. For an intensive review of this subject the reader is referred to the works of Hansen, Lindner, Guilliermond, Tanner, and others.

Nutrition of Yeasts.—Carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, magnesium, calcium, sulphur, and chlorine are desirable elements in the nutrition of yeasts.

Carbon.—In considering sugars as carbon sources, one must be reminded that the ability of a yeast to assimilate a sugar may be quite different from its ability to ferment the same sugar. Likewise the ability to assimilate a given compound varies with different varieties of yeasts.

Carbon may be supplied in the form of sugars, aldehydes, salts of some organic acids, glycerol, or ethanol.

Guilliermond has stated that impure maltose is best suited to the metabolism of yeasts.[1] Sucrose, glucose, fructose, and raffinose are not so important from the viewpoint of assimilation, yet some of these sugars are readily fermented. Lactose is assimilated only in certain isolated cases.

Acetates, citrates, lactates, malates, succinates, and tartrates, as well as lactic, malic, succinic, and tartaric acids, may be used as sources of carbon.

Ethyl alcohol, in low concentration, occasionally may be utilized.

Nitrogen.—Nitrogen may be supplied from a number of sources. Soluble protein degradation products—peptones, peptides, and amino acids; amides; urea; ammonium salts and, under certain conditions, other compounds may be used as sources of nitrogen. Ammonium sulphate, phosphates, or chloride are frequently used in semisynthetic media and in production of compressed yeast. Nitrates are rarely assimilable by yeasts.

Mineral Elements.—Magnesium, phosphates, potassium, sulphur, calcium, and chlorine, if used, are supplied as salts. Potassium phosphates, magnesium sulphate, and calcium phosphate or chloride are the salts commonly employed.

The composition of some semisynthetic media is given in Table 5.

Yeasts grow best in media containing an abundance of assimilable sugar and other suitable nutrient substances that have acid reactions.

[1] Guilliermond, A., "The Yeasts," translated by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

In the laboratory, many yeasts grow well on liquid or solid malt media, on dextrose agar, in beer wort, or on semisynthetic media of the nature outlined above. Natural media, such as the expressed juice of grapes, apples, pears, or other fruit juices, are excellent, since sugars are present along with nitrogen-containing compounds and dissolved salts at a pH favorable for development.

TABLE 5.—SOME SEMISYNTHETIC MEDIA

Substance	Medium of Devereux and Tanner,[1] grams	Medium F of Fulmer and Nelson,[2] grams	Medium of Mayer,[3] grams
Sucrose	10.0	10.0	15.0*
Dextrin	.....	0.60	
NH_4Cl	0.12	0.188	
K_2HPO_4	0.05	0.100	
KH_2PO_4	.....	.....	0.1
$CaCl_2$	0.01	0.100	
$Ca_3(PO_4)_2$	.....	.....	0.1
$MgSO_4$	0.02	.....	0.1
Distilled water	100.00 cc.	100.0 cc.	100.0 cc.

[1] DEVEREUX, E. D., and F. W. TANNER, *Jour. Bact.*, **14**: 317 (1927).

[2] Formula for incubation at 30°C. F. W. Tanner, E. D. Devereux, and F. M. Higgins, *Jour. Bact.*, **11**: 45 (1926).

[3] GUILLIERMOND, A., "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

* Candied sugar.

Media favorable for the growth of yeasts are also suitable for the growth of molds. Owing to the rather acid nature of these media many bacteria fail to develop well in them or are inhibited entirely.

For a further discussion of the subject of yeast nutrition, the reader is referred to Guilliermond's or other standard texts.

Relative Rates of Fermentation of Glucose and Fructose.—Not all sugars are fermented at the same rate. Nor do all yeasts act with equal efficiency. In low concentrations of sugar, fructose is fermented at a slower rate (expressed as milligrams of carbon dioxide per minute) than glucose by brewer's yeast at 30°C.[1] Although the maximum rates for the fermentation of these two sugars differ but little, the rate for glucose is slightly greater than that for fructose at the same concentration. Glucose is fermented at about the same rate in concentrations between 1 and 10 per cent; fructose, between 2 and 8 per cent. At high concentrations of the sugars, the rate of fermentation of fructose is less than that of glucose. This is probably referable to the difference in the ease

[1] HOPKINS, R. H., and R. H. ROBERTS, Kinetics of Alcoholic Fermentation of Sugars by Brewer's Yeast, *Biochem. Jour.*, **29**: 931 (1935).

with which the enzymes involved can bring about the cleavage of the sugar molecule.

Yeast Enzymes.—The enzymes present in yeast include, usually, at least three different groups: those concerned with respiration; those concerned with breaking sugars down to the hexose stage, for example, maltase and invertase; and finally those concerned with fermentation.

Enzymes catalyze the complex chemical changes that take place in nutrient sugar solutions. Whether or not a carbohydrate is fermented or assimilated depends on the nature of the enzymes elaborated by the yeast, provided, of course, that conditions are otherwise favorable for fermentation or growth. Polysaccharides, in general, are not fermented. Lactose is fermented by *Saccharomyces fragilis* (*S. kefir*), the yeast of the fermented milk product, kefir, and by a few other yeasts but not by *S. cerevisiae* and *S. ellipsoideus*, which represent the best-known and probably most widely distributed types of yeast.

The enzymes of yeasts are of two kinds: endoenzymes (intracellular) and exoenzymes (extracellular). These enzymes react according to the general laws governing enzyme reactions but show also a degree of specificity in each case.

The enzymes of yeasts may be classified as hydrolases and desmolases.

Hydrolases are enzymes that convert carbohydrates, proteins, and esters to simpler substances by the addition of water followed usually by cleavage. For example, sucrose is converted, after the absorption of water, to 1 molecule each of glucose and fructose as a result of the action of sucrase, an enzyme; while raffinose, a trisaccharide, is converted to 1 molecule each of fructose, glucose, and galactose through the action of melibiase and sucrase, enzymes possessed by "bottom" yeast.

Glycogen is hydrolized to glucose, but no cleavage follows the addition of the molecule of water to the glycogen molecule. Although glycogenase may thus convert glycogen to glucose, it may, under certain conditions, cause the reaction to go in the opposite direction. Thus, some enzymes have the ability to catalyze reversible reactions and so to synthesize compounds as well as to break them down to simpler components.

Desmolases are enzymes or enzyme complexes involved in respiration and metabolism. Zymase is a desmolase of intracellular origin and rarely or never passes through the cell wall into the surrounding medium.

Zymase is not a single enzyme, but a complex of enzymes and coenzymes. The term "holozyme" has been coined to include the zymase complex plus all its activators. Hexokinase, oxidoreductase, enolase, carboxylase, and phosphatase are said to be some of the enzymes of this complex.

Table 6 summarizes data concerning some of the enzymes found in yeasts, the substrates acted upon, and the end products formed as a result of this action.

TABLE 6.—SOME ENZYMES OF YEASTS, SUBSTRATES ACTED UPON, AND PRODUCTS FORMED

Enzyme	Substrate	Products formed
I. Hydrolases:		
A. Carbohydrases.		
1. Sucrase (Saccharase, invertase, invertin)	$C_{12}H_{22}O_{11}$ Sucrose	$C_6H_{12}O_6$ Glucose + $C_6H_{12}O_6$ Fructose
2. Maltase	$C_{12}H_{22}O_{11}$ Maltose	$2C_6H_{12}O_6$ Glucose
3. Lactase	$C_{12}H_{22}O_{11}$ Lactose	$C_6H_{12}O_6$ Galactose + $C_6H_{12}O_6$ Glucose
4. Melibiase	$C_{12}H_{22}O_{11}$ Melibiose	$C_6H_{12}O_6$ Galactose + $C_6H_{12}O_6$ Glucose
5. Trehalase	$C_{12}H_{22}O_{11}$ Trehalose	$2C_6H_{12}O_6$ Glucose
6. Glycogenase	$(C_6H_{10}O_5)_x$ Glycogen	$C_6H_{12}O_6$ Glucose
	or $C_6H_{12}O_6$ Glucose	$(C_6H_{10}O_5)_x + xH_2O$ Glycogen
B. Proteolytic enzymes:		
1. Proteases	Yeast proteins	Proteoses, peptones, and polypeptides
2. Peptidases	Peptides	Amino acids
C. Esterases:		
1. Phosphatases:		
a. Polynucleotidase	Nucleic acid	Mononucleotides
b. Phosphatese	Hexose + H_3PO_4	Hexosephosphate
D. Amidases:		
1. Asparaginase	$H_2N{\cdot}CO{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH$ Asparagine	$HOOC{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH + NH_3$ Aspartic acid
II. Desmolases:		
A. Zymase group:		
1. Oxydoreductase (Mutase, dehydrase)	RCHO Aldehyde	RCH_2OH Alcohol + RCOOH Acid
2. Glycerolphosphoric dehydrogenase	Glycerolphosphoric acid	Glyceraldehyde phosphoric acid
3. Carboxylase	$CH_3{\cdot}CO{\cdot}COOH$ Pyruvic acid	$CH_3{\cdot}CHO$ Acetaldehyde + CO_2 Carbon dioxide
4. Methylglyoxalase	$CH_3CO{\cdot}CHO$ Methylglyoxal	$CH_3CHOHCOOH$ Lactic acid
5. Hexokinase	Hexoses	Active hexoses

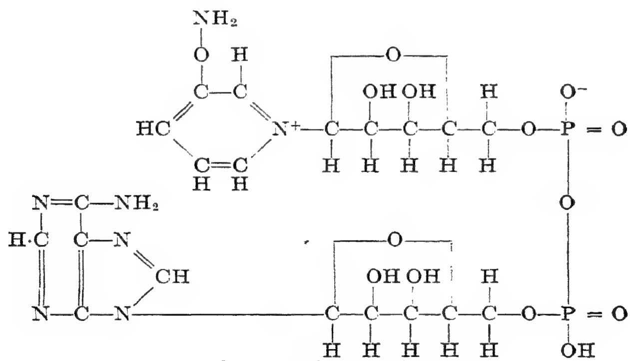
Coenzymes and Activators.—A coenzyme, phosphate, magnesium, or some other substance may be essential in order that an enzyme may function properly. Cozymase (coenzyme I) is required for the action of oxidoreductase; cocarboxylase, for carboxylase, the enzyme which splits carbon dioxide from such alpha-ketonic acids as pyruvic acid. Magnesium is essential for the activation of phosphatase.[1] Glutathione is required by methylglyoxalase.

Cell-free yeast juice from crushed or autolyzed cells will ferment sugar but more slowly than yeast cells, a fact demonstrated by Buchner. If yeast juice is dialyzed by passing it through a semipermeable membrane, neither the dialyzate, the portion passing through the membrane, nor the residue will produce fermentation of sugar. Should the two fractions be mixed, however, fermentation ensues.

The residue, the portion not passing through the semipermeable membrane, is thermolabile, being destroyed by boiling. The non-dialyzable enzymes are found in this fraction.

Cozymase.—The dialyzate is thermostable and contains, in addition to other substances, cozymase or coenzyme I.

Euler and Schlenk[2] have proposed the following structural formula for cozymase or coenzyme I.



Cozymase (Coenzyme I)

Euler and others believe that cozymase is a hydrogen-carrying coenzyme, which picks up 2 atoms of hydrogen (to form dihydrocozymase) and later donates them. It acts as an intermediate between two apo-dehydrases in the transportation of hydrogen from the donator system (glyceraldehyde phosphoric acid-phosphoglyceric acid) to the acceptor

[1] TAUBER, H., "Enzyme Chemistry," John Wiley & Sons, Inc., New York, 1937.

[2] LOHMANN, K., The Chemistry and Metabolism of the Compounds of Phosphorus, *Ann. Rev. Biochem.*, **7**: 125 (1938).

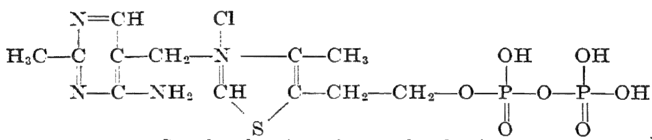
system (acetaldehyde-ethyl alcohol) in the ethanol fermentation.[1] (For further details concerning the function of cozymase, refer to page 79.)

Cozymase may be prepared by dialyzing fresh yeast juice, which contains about 0.5 g. of cozymase per kilogram of juice,[2] or by washing zymin with water.

The coenzyme may be isolated from most animal tissues; from many plants and fungi; from certain bacteria, namely, those giving rise to propionic and lactic acids; and from red blood corpuscles.

The Adenylic Acid System.—The dialyzate of yeast juice also contains a phosphate carrier system, which functions as a coenzyme. This system is made up of adenosinemonophosphate (adenylic acid), adenosinediphosphate and adenosinetriphosphate. For further details, consult the chapter on Facts and Theories Concerning the Mechanism of the Ethanol Fermentation.

Cocarboxylase.—Lohmann and Schuster[3] have assigned the following structural formula to the coenzyme cocarboxylase, which is diphosphorylated vitamin B:



Cocarboxylase (aneurin pyrophosphate)

Cocarboxylase is thermostable and is found in yeast juice. When beer yeast is washed with an alkali, it can no longer decarboxylate pyruvic acid, but the addition of boiled, fresh beer-yeast juice causes the system to become active again, according to the researches of Anhagen,[4] who called the thermostable principle "cocarboxylase."

Phytochemical Reduction.—Yeast possesses strong reducing properties.

A relatively large number of substances may be added to an actively fermenting medium, containing yeast and sugar, and become reduced. Aldehydes are reduced to their corresponding alcohols; for example, acetaldehyde, glyceraldehyde, furfuraldehyde (furfural), and benzaldehyde are reduced to ethyl alcohol, glycerol, furfuralcohol, and benzyl alcohol, respectively. Ketones and diketones may be reduced to their

[1] Euler, H. von, *Angew. Chem.*, **50**: 831 (1937).

[2] Meyerhof, O., und P. Ohlmeyer, *Biochem. Zeit.*, **290**: 334 (1937).

[3] Lohmann, K., und P. Schuster, *Naturwissenschaften*, **25**: 26 (1937); *Biochem. Zeit.*, **294**: 188 (1937).

[4] Anhagen, E., *Zeit. physiol. Chem.*, **204**: 149 (1932); *Biochem. Zeit.*, **258**: 330 (1933).

corresponding secondary alcohols, likewise, but the rate of reduction is slower than it is for aldehydes.[1]

The reducing action of yeast is not confined, however, to aldehydes and ketones alone, for methylene blue may be reduced; aniline may be formed from nitrobenzene, and hydrogen sulphide from sulphur. Other types of substances may become reduced also.

Reduction may be readily demonstrated by adding the substance to be reduced to a fermenting medium containing 10 per cent sucrose and 10 per cent living yeast.

Classification of Yeasts.—The problem of classification of the yeasts is one of great difficulty, and modern work has not tended to eliminate all the confusion that has previously existed. The most recent classification, here given, supersedes that published in Guilliermond's treatise on this group of fungi but agrees with it in putting practically all the yeasts now industrially important in the genus *Saccharomyces*.

The following classification is that of Stelling-Dekker, modified in some respects by Guilliermond.[2]

Family of Endomycetaceae

I. Subfamily of the *Eremascoideae*.

Genus *Eremascus* Eidam.

Thallus formed by a mycelium without any form of asexual multiplication. Ascs formed by isogamic conjugation, 8 round ascospores.

II. Subfamily of the *Endomycetoïdeae*.

Thallus formed by a typical mycelium multiplying by means of oïdia or thallus reduced to the state of oïdia.

1. Genus *Endomyces* Reess.

Typical mycelium multiplying by oïdia.

Ascs formed by heterogamic conjugation or by parthenogenesis; 4 round or hat-shaped ascospores.

2. Genus *Schizosaccharomyces* Lindner.

Thallus reduced to the state of oïdia. Ascs formed by isogamic conjuation; 4 to 8 round ascospores.

III. Subfamily of the *Saccharomycoideae*.

Thallus formed by a typical mycelium multiplying by means of conidial buds and occasionally at the same time by oïdia, or thallus reduced to the state of yeast cells.

A. Tribe of the *Endomycopseae*.

Thallus formed by a typical mycelium multiplying by means of conidia yeasts (conidial buds) and occasionally at the same time by oïdia. Ascs formed by heterogamic conjugation or by parthenogenesis, occasionally after rudimentary attempts of

[1] Harden, A., "Alcoholic Fermentation," 4th ed., Longmans, Green & Company, New York, 1932.

[2] Guilliermond, A., La sexualité, le cycle de développement, la phylogénie, et la classification des levures d'après les travaux récents, Masson et Cie, Paris, 1937.

union (vestiges of sexuality). Ascospores in the form of a hat, sickle, or girdled in the middle by a projecting filament, smooth or sometimes verrucose.

1. Genus *Endomycopsis* Dekker.

B. Tribe of the *Saccharomyceteae.*

Thallus reduced to yeast cells that are able occasionally to produce rudimentary mycelia.

1. Genus *Zygosaccharomyces* Barker.

Cells round, oval, or elongated. Ascs formed by iso- or heterogamic conjugation; 1 to 4 round or reniform ascospores. Fungi developing in liquid media first in the form of a deposit and producing a pellicle or ring only slowly or not producing any. Producers of alcoholic fermentations.

2. Genus *Torulaspora* Lindner.

Round cells. Ascs formed by parthenogenesis in cells having tried to conjugate; 1 to 2 round and smooth ascospores. Fungi vegetating in the form of a deposit and not producing pellicle or ring or producing it only slowly.

3. Genus *Saccharomyces* Meyen.

Round, oval, or elongated cells, at times with rudimentary mycelia. Ascs formed without conjugation; 1 to 4 round and smooth ascospores. Conjugation may be produced between the ascospores or more generally between the first cells produced by their budding; zygospores being the point of departure from diploid cells which, after having produced numerous generations, are transformed into ascs. Fungi vegetating first in liquid media in the form of a deposit. Film or ring formed slowly or not at all. Producers of fermentations.

4. Genus *Pichia* Hansen.

Cells oval or elongated. Ascs formed by iso- or heterogamic conjugation or by parthenogenesis; 1 to 4 hemispherical, reniform, or triangular smooth ascospores. Fungi forming, from the beginning, a film on the surface of liquid media. Oxidations, sometimes fermentations.

a. Subgenus *Zygopichia* Klöcker.

Ascs derived from iso- or heterogamic conjugation.

b. Subgenus *Pichia* Hansen.

Ascs formed by parthenogenesis.

5. Genus *Hansenula* (*Willia* Klöcker) Sydow.

Yeasts oval or elongated, rarely round, at times rudiments of mycelium. Ascs formed without conjugation; 1 to 4 ascospores having the appearance of a hat or girdled with a protruding ring. In certain forms (*Hansenula Saturnus*), conjugation among the ascospores, or more generally among the first cells derived from their budding, and producing zygospores, point of departure of numerous generations of diploid cells being transformed finally into ascs. Fungi developing from the beginning on liquid media, without film formation. Oxidations and sometimes fermentations.

6. Genus *Debaryomyces* Klöcker.

Round or oval cells, sometimes rudimentary mycelia. Ascs formed by iso- or heterogamic conjugation; 1 to 2, sometimes 4, round and globular ascospores. On liquid medium, first a deposit, then film or ring. Sometimes producers of fermentations.

7. Genus *Schwanniomyces* Klöcker.

Round or oval cells, occasionally rudimentary mycelia. Ascs formed by parthenogenesis after attempts of union (vestiges of sexuality); 1 to 2 ascospores provided with a medium of a projecting filament and with a verrucose wall.

C. Tribe of *Nadsonieae.*

Cells elongated and ordinarily apiculate and producing new cells at either pole by a process intermediate between budding and division.

1. Genus *Nadsonia* Sydow (*Guilliermondia* Nadson and Konokotine).
   Ascs formed in a bud derived from a zygote resulting from a heterogamic conjugation; 1 to 2, sometimes up to 4, round and verrucose ascospores. Vegetation on liquid medium, in form of a deposit and with production of alcohol.
2. Genus *Saccharomycodes* Hansen.
   Ascs with 4 round and smooth ascospores. Conjugation produced regularly between the ascospores. Vegetation in liquid media first in form of a deposit. Produce fermentations.
3. Genus *Hanseniaspora* Zikes (*Hansenia* Lindner).
   Cells very sharply apiculated. Ascs formed without conjugation; 1 to 4 hemispherical or round ascospores.

IV. Subfamily of *Nematosporoideae*.

Yeasts of variable forms and often mycelium. Ascs with 1 to 8 ascospores in form of long needles.

1. Genus *Monosporella* Keilin (*Monospora* Metchnikoff).
   Oval yeasts. Ascs with 1 ascospore in form of long needle.
2. Genus *Nematospora* Peglion.
   Yeasts of variable form and mycelium; 1 to 8 ascospores in form of needles provided at one of their poles with a sort of long flagellum.
3. Genus *Coccidiascus* Chatton.
   Oval yeasts. Ascs appearing to be derived from an isogamic conjugation; 8 ascospores in the form of long spindles.

In the discussions and descriptions of processes that will be considered in this book, only those types of yeast which are of importance industrially or in their relation to deterioration processes in foods, drinks, etc., will be taken up in detail.

GROWTH-PROMOTING SUBSTANCES

Definition.—Growth-promoting substances, or growth-accessory factors, may be considered to be substances which, when added to a medium containing the usual sources of energy, carbon, nitrogen, and basic inorganic salts, accelerate the growth of a microorganism.

Controversial Nature.—The subject of growth-promoting substances, especially bios, has been a cause of controversy, particularly during the first part of the present century. Failure to agree on findings in different laboratories has been due in large part to the use of different species or strains of yeasts or other microorganisms. Copping,[1] Williams,[2] and others have shown that the requirement for bios, for example, depends on the type of yeast and the composition of the medium.

Bios.—Space does not permit an extensive review of the literature concerning bios. The subject was reviewed, however, by Tanner,[3] in

[1] Copping, A. M., *Biochem. Jour.*, **23**: 1050 (1929).

[2] Williams, R. J., J. L. Wilson, and F. W. von der Ahe, *Jour. Am. Chem. Soc.*, **49**: 227 (1927).

[3] Tanner, F. W., *Chem. Rev.*, **1**: 397 (1925).

1925; by Lutman[1] in 1929; by Buchanan and Fulmer[2] and by Miller[3] in 1930.

Since the appearance of these reviews, considerable new research has been carried out concerning the growth substances of yeasts, and the identities of several components of bios have been established.

In 1935, Thimann[4] outlined the more important advances in the knowledge of bios, while Koser and Saunders[5] (1938) presented an extensive review concerning bacterial accessory growth factors, which included a survey of yeasts and molds.

Origin of the Term.—Wildiers[6] (1901) proposed the name "bios" for the hypothetical organic substance of biological origin that stimulated the growth of yeasts. He based his evidence for the existence of bios on a series of observations made during his researches. He observed that a beer yeast, a strain of *Saccharomyces cerevisiae*, grew poorly on a synthetic medium that contained ammonia as the source of nitrogen, but that the addition of a small amount of organic material, such as beer wort, caused rapid growth of the yeast. Likewise small amounts of inoculum sometimes failed to produce growth in a medium, whereas relatively large quantities of inoculum produced rapid growth. The filtrate from boiled yeast produced the same effect as a large inoculum. Liebig's meat extract and other organic substances of biological origin stimulated growth.

Wildiers's ideas on bios were soon challenged, but they served as a basis for subsequent important research.

Chemistry of Bios.—The complex known as bios is soluble in water and in 80 per cent alcohol but insoluble in absolute alcohol and ether. It is relatively stable to heat and moderately so to acids but is destroyed by boiling in a 20 per cent solution of sulphuric acid. A boiling solution of sodium hydroxide, of concentration much in excess of 1 per cent, usually destroys bios. It is dialyzable through a semipermeable membrane.[7]

Lucas[8] demonstrated that bios may be separated into two fractions by the use of an alcoholic solution of barium hydroxide. The barium salt of one fraction, bios I, was insoluble in alcohol, while no salt was

[1] ...N, B. F., "Microbiology," McGraw-Hill Book Company, Inc., 1929.

[2] Buchanan, R. E., and E. I. Fulmer, "Physiology and Biochemistry of Bacteria," Vol. II, Williams & Wilkins Company, Baltimore, 1930.

[3] Miller, W. L., *Jour. Chem. Education*, **7**: 263 (1930).

[4] Thimann, K. V., *Ann. Rev. Biochem.*, **4**: 545 (1935).

[5] Koser, S. A., and F. Saunders, *Bact. Rev.*, **2**: 122 (1938).

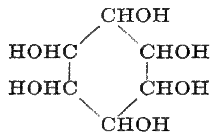
[6] Wildiers, E., *Cellule*, **18**: 313 (1901).

[7] Anderson, C. G., "An Introduction to Bacteriological Chemistry," The Williams & Wilkins Company, Baltimore, 1938.

[8] Lucas, G. H. W., *Jour. Phys. Chem.*, **28**: 1180 (1924).

formed by the second fraction, which was designated as bios II. Neither of the fractions by itself had much activity, but a combination of the two fractions produced growth stimulation.

1. *Bios I.* *i-Inositol.*—Eastcott[1] identified bios I as optically inactive inositol, which has the following structural formula:



i-Inositol

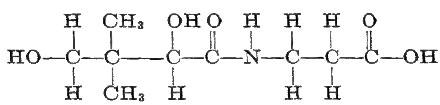
Inositol had very little action when used alone but was effective when combined with other components of bios.

2. *Bios II.*—Bios II, the portion of bios not precipitated by an alcoholic solution of barium hydroxide, has been fractionated into other substances by different methods.

a. PANTOTHENIC ACID.—Using fuller's earth, Williams and his coworkers[2] separated bios II into an adsorbed fraction, replaceable by vitamin B_1 (thiamin or aneurin), and an unadsorbed fraction designated by them as "pantothenic acid."

The term "pantothenic" is derived from Greek and signifies "from everywhere." Pantothenic acid is thus named, because it may be isolated from a large number of sources, which include plant (bacteria, slime molds, etc.) and animal tissues.

Pantothenic acid has been synthesized by Stiller, Keresztesy, and Finklestein[3] of the Merck Research Laboratories. It has the following chemical structure:



It is prepared by condensing alpha-hydroxy-beta, beta-dimethyl-gamma-butyrolactone, a product that has been synthesized, with beta-alanine.

Small amounts of thiamin, or relatively large quantities of *i*-inositol, or mixtures of the two, increased the stimulating effect of this acid in respect to certain yeasts.[4]

[1] EASTCOTT, E. V., *Jour. Phys. Chem.*, **32**: 1094 (1928).
[2] WILLIAMS, R. J., and E. BRADWAY, *Jour. Am. Chem. Soc.*, **53**: 783 (1931); and HONN, *Jour. Plant Physiol.*, **7**: 629 (1932).
[3] WILLIAMS, R. J., and R. T. MAJOR, *Science*, **91**: 246 (1940).
[4] WILLIAMS, R. J., and D. H. SAUNDERS, *Biochem. Jour.*, **28**: 1887 (1934).

b. BIOTIN.—If bios is fractionated with charcoal, the adsorbed portion contains a substance designated by Kögl and his associates[1] as "biotin." This substance may be eluted from charcoal by the aid of an aqueous solution of ammonia and acetone. Afterward the product may be purified. Kögl[2] states that approximately 360 tons of ordinary yeast would be necessary for the production of 1 g. of biotin. The unadsorbed portion has been called bios III.

Biotin has been assigned the tentative formula $C_{11}H_{18}O_3N_2S$. It has been obtained as the methyl ester in crystalline form. One part of biotin in 4×10^{10} parts of medium caused a distinct stimulation, while one part in 10^{11} parts produced a perceptible effect on the growth of yeast (strain *M*). The growth effect is increased by the presence of the cofactors *i*-inositol and thiamin.

c. BETA-ALANINE.—Miller and his associates[3] have separated bios II into 2 fractions by the use of charcoal. The portion adsorbed by the charcoal and removed by shaking with an aqueous solution of acetone and ammonia was designated as "bios II*B*," while the unadsorbed portion of bios II, the filtrate, was called "bios II*A*." The properties of bios II*A* are due to beta-alanine and leucine.[4]

In a medium containing sugar, salts, and 5 mg. of inositol per liter, the growth of several strains of yeasts was stimulated by the addition of as little as 0.08 microgram of β-alanine per cubic centimeter of medium (approximately 1 part in 12,000,000). Aspartic acid enhanced the effect.[5] Pantothenic acid, and thiamin in at least one case, stimulated growth when added.

At very high dilutions, β-alanine may be slightly inhibitory.[6] Nielsen and Hartelius state that the β-alanine is toxic to yeast except when asparagine, or aspartic acid, is a constituent of the medium.[7]

d. THIAMIN.—Williams and his associates (1930) observed that thiamin (vitamin B_1, or aneurin) stimulated the growth of a certain strain of yeast.[8]

Thiamin has the following structural formula:

F., und B. TÖNNIS, *Zeit. physiol. Chem.*, **242**: 43 (1936); *Chem. & Ind.*, **57**: 49 (1938).

[3] MILLER, W. L., E. V. EASTCOTT, and E. M. SPARLING, *Trans. Roy. Soc. Can.*, III, **26**: 165 (1932).

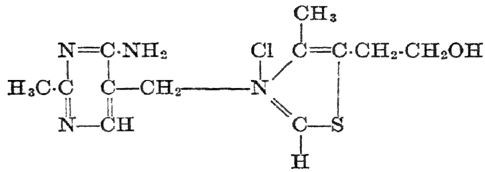
[4] MILLER, W. L., *Trans. Roy. Soc. Can.*, III, **30**: 99 (1936).

[5] WILLIAMS, R. J., and E. ROHRMAN, *Jour. Am. Chem. Soc.*, **58**: 695 (1936).

[6] WILLIAMS and ROHRMAN, *ibid.*

[7] NIELSEN, N., und HARTELIUS, *Biochem. Zeit.*, **296**: 359 (1938).

[8] WILLIAMS, R. J., and R. R. ROEHM, *Jour. Biol. Chem.*, **87**: 581 (1930).



Pyrimidine ring Thiazole ring

Farrell[1] found that *Saccharomyces hanseniospora valbyensis* reproduced well in a medium containing tomato juice but not in a medium containing inositol, bios II*A*, and bios II*B*. She designated the substance that stimulated growth as bios V. It has been shown that bios V may be replaced by thiamin.[2]

Thiamin has a marked effect on yeast fermentation, according to Schultz and his coworkers.[3]

Thiamin, derivatives of thiamin, and the pyrimidine and/or the thiazole portions of the thiamin molecule may serve as accessory growth factors for certain microorganisms.[4]

Rhodotorula rubra and *R. flava*[5] are stimulated not only by the intact thiamin molecule but also by the pyrimidine ring of the molecule. A concentration of approximately 0.4 microgram of thiamin in 25 cc. of medium produced maximum growth.

The action of thiamin, pyrimidine, and thiazole on various yeasts has been investigated by Schultz and his associates.[6]

e. BIOS VII.—A "useful constituent," which accompanies bios II*B*, has been named "bios VII" by Miller.[2]

Sources of Bios Constituents.—Constituents of bios may be isolated from a large number of sources: from wild yeasts, from *Aspergillus niger*,[7] from the leaves and buds of the birch,[8] from malt rootlets,[9] from beer wort, from tomato juice, from the charcoal used in the refining of raw brown sugar, and from many other sources.

[1] FARRELL, L. N., *Trans. Roy. Soc. Can.*, III, **29**: 167 (1935).

[2] MILLER, W. L., *Trans. Roy. Soc. Can.*, III, **31**: 159 (1937).

[3] SCHULTZ, A. S., L. ATKIN, and C. N. FREY, *Jour. Am. Chem. Soc.*, **59**: 948 (1937).

[4] KOSER, S. A., and F. SAUNDERS, *Bact. Rev.*, **2**: 99 (1938).

[5] SCHOPFER, W. H., *Compt. rend.*, **205**: 445 (1937); *Compt. rend. soc. biol.*, **126**: 842 (1937).

[6] SCHULTZ, A. S., L. ATKIN, and C. N. FREY, *Jour. Am. Chem. Soc.*, **60**: 490 (1938).

[7] NIELSEN, N., und V. HARTELIUS, *Compt. rend. trav. lab. Carlsberg, Sér. Physiol.*, **22**: 1 (1937).

[8] DAGYS, J., *Protoplasma*, **24**: 14 (1935).

[9] DEAS, J., *Jour. Biol. Chem.*, **61**: 5 (1924).

Function of Growth Substances.—Thiamin is closely related to cocarboxylase and is believed to function in the structure of the latter compound.

The exact functions of biotin, inositol, β-alanine, and pantothenic acid are not known at present.

For further information concerning this subject, the reader is referred to the publications listed in the following bibliography, some of which contain additional references.

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Dagys, J.: Die Hefewuchsstoffe in Knospen und Blättern, *Protoplasma*, **26**: 20 (1936).

Devloo, R.: Purification du bios de Wildiers, *Cellule*, **23**: 361 (1906).

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CHAPTER III

THE PRODUCTION OF INDUSTRIAL ALCOHOL BY FERMENTATION

One of the most important and best-known industrial fermentations is that in which ethyl alcohol is produced from sugars by yeasts. The chemical manufacturer, the brewer, the distiller, the baker, the vinegar manufacturer, the scientist, the housewife, and many others depend in one way or another on the ability of the yeast to convert sugars to alcohol, carbon dioxide, and other end products. Since low-priced and waste carbohydrate materials may be used in the manufacture of ethyl alcohol, the fermentation process has not only great present but also vast potential value.

The subject of ethyl alcohol production by fermentation has assumed new interest on account of attempts to find substitutes for gasoline. Blends of alcohol with gasoline, especially a blend containing 10 per cent ethyl alcohol, may be used satisfactorily in the present internal-combustion type of motor. Present-day demands for motor fuels are great, but the sources of petroleum are limited.

Definitions.—Ethyl alcohol (CH_3CH_2OH), or ethanol, may be referred to by other names. For example, the alcohol may be named to indicate the source of raw material from which it is manufactured or to indicate the general purpose for which it is to be used. Grain alcohols are alcohols made from grains, such as corn, wheat, or rice. The term "grain alcohol" is sometimes used to signify ethyl alcohol in contrast to methyl alcohol (CH_3OH), or methanol, which is manufactured by the destructive distillation of wood, by synthesis, or by other means. Molasses alcohol is alcohol produced from sugar-cane molasses. Industrial alcohol is ethyl alcohol used for industrial purposes. Under this term is included power, or fuel alcohol, *i.e.*, alcohol used in combination with gasoline or other motor fuels.

Production Statistics.—The quantity of alcohol produced in the industrial-alcohol plants of the United States and the number of plants operated during the fiscal years 1920–1937 are shown in Table 7.

Over 88 per cent of the entire quantity of industrial alcohol manufactured during the fiscal year 1937 was produced by the seven states listed in Table 8.

Raw Materials.—Ethyl alcohol may be produced from any fermentable sugar by yeasts under suitable conditions. Since starches and certain other carbohydrates may be hydrolyzed to fermentable sugars by biological or chemical means, there are available many possible sources of sugar.

TABLE 7.—QUANTITY OF ALCOHOL PRODUCED AT INDUSTRIAL ALCOHOL PLANTS FROM 1920 TO 1937[1]

Fiscal year[2]	Plants operated	Quantity produced, proof gallons[3]	Fiscal year[2]	Plants operated	Quantity produced, proof gallons[3]
1920	37	18,933,551	1929	52	200,832,051
1921	70	85,068,776	1930	50	191,859,342
1922	73	79,906,101	1931	46	166,014,346
1923	76	122,402,850	1932	43	146,950,913
1924	70	135,897,726	1933	34	115,609,754
1925	71	166,165,518	1934	34	165,103,582
1926	64	202,271,670	1935	32	180,645,920
1927	62	184,323,017	1936	35	196,126,236
1928	55	169,149,905	1937	38	223,181,228

[1] From reports of the U.S. Dept. of the Treasury, in P. B. Jacobs and H. P. Newton, *U.S. Dept. Agr.*, *Misc. Pub.* 327, December, 1938.
[2] Ended June 30.
[3] Proof gallons divided by 1.9 equals U.S. wine gallons of 95 per cent concentration.

TABLE 8.—PLANTS AND WAREHOUSES OPERATED AND ETHYL ALCOHOL PRODUCED BY SEVEN LEADING STATES DURING FISCAL YEAR 1937[1]

State	Plants operated[2]	Warehouses operated[2]	Quantity produced, proof gallons
Pennsylvania	3	7	49,507,373
New Jersey	3*	5	41,764,964
Louisiana	4	6	31,203,855
Maryland	1	3	26,053,152
Indiana	4	4	18,014,606
West Virginia	1	1	17,493,652
Illinois	3	9	10,991,274
Total (United States including Hawaii and Puerto Rico)	38	73	223,181,228

[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue*, 1937.
[2] Number operated during any part of the year.
* One experimental plant.

1. *Types.*—Raw materials may be classified into three principal types: (*a*) the saccharine materials, such as sugar cane, sugar beets, molasses, and fruit juices; (*b*) the starchy materials, which include the cereals (corn, malt, barley, oats, rye, wheat, rice, grain sorghum, and the like),

TABLE 9.—PERCENTAGE OF TOTAL ETHYL ALCOHOL PRODUCED FROM DIFFERENT KINDS OF RAW MATERIALS, 1932–1937[1]

Raw material	1932, per cent	1933, per cent	1934, per cent	1935, per cent	1936, per cent	1937, per cent
Molasses, blackstrap	84.76	83.00	83.40	85.49	76.15	75.73
Ethyl sulphate, synthetic	9.69	9.70	7.34	9.73	16.06	15.18
Grain	3.75	4.13	6.29	2.74	7.04	8.36
Pineapple juice	......	......	0.42	0.34	0.32	0.21
Fermented liquor	......	......	0.11	0.07	0.03	
Corn sirup	......	......	0.02	......	0.03	
Mixtures (grain, hydrol, molasses)	0.86	2.24	2.42	0.60	0.37	0.51
Other raw materials	0.94	0.93	......	......	......	0.01
Hydrol	......	......	......	1.03		
Total	100.00	100.00	100.00	100.00	100.00	100.00

[1] From reports of Alcohol Tax Unit, in Jacobs and Newton, *op. cit.*

TABLE 10.—ETHYL ALCOHOL PRODUCTION, BY KINDS OF MATERIALS USED, FOR FISCAL YEAR 1937[1]

Kind of material	Quantity used		Alcohol produced	
	Amount	Unit	Proof gallons	Per cent of total
Molasses[2]	201,792,913	Gallons	169,013,295	75.73
Ethyl sulphate	25,492,675	Gallons	33,889,083	15.18
Grain[2]	225,623,538†	Pounds	18,651,946	8.36
Pineapple juice	3,598,222	Gallons	459,726	0.21
Corn sirup	28,446	Gallons	30,991	0.01
Cider	34,820	Gallons	4,025	[3]
Fermented liquor	23,157	Gallons	2,945	[3]
Mixtures:				
Molasses	838,143	Gallons	1,129,216*	0.51
Sulphite liquor	12,060,125	Gallons		
Grain	7,053,649	Pounds		
Manioca meal	634,552	Pounds		
Sweet potatoes	32,099	Pounds		
Sorgo	5,930	Pounds		
Dry feed	1,161	Pounds		
Tankage	475	Pounds		
Total	....	....	223,181,227	100.00

* Includes 23,184 proof gallons produced at experimental plants from materials not shown.
† Includes 19,180 lb. of diamalt.
[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue,* 1937.
[2] Additional amounts used in combination with other materials included under "Mixtures."
[3] Less than 0.01 per cent.

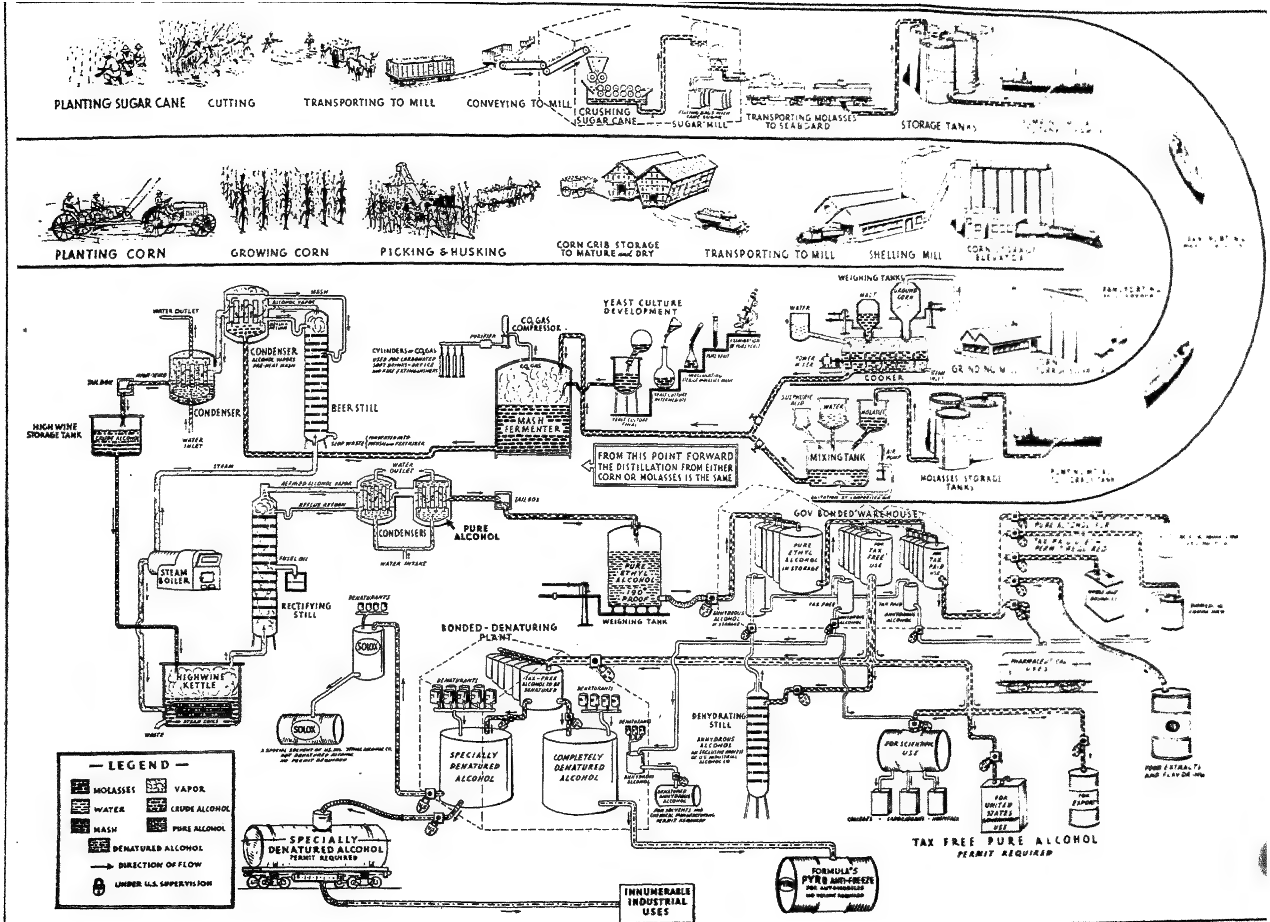


FIG. 6.—Flow sheet of industrial ethyl alcohol manufacture. (*Courtesy of the U.S. Industrial Chemicals, Inc., New York City.*)

potatoes, sweet potatoes, Jerusalem artichokes (girasol), manioca meal, and other substances; and (*c*) cellulosic materials, such as wood and waste sulphite liquor.

2. *Principal Raw Materials Utilized.*—In the United States, molasses is the principal raw material used in the production of industrial alcohol. Table 9 gives information concerning the percentages of the various raw materials used to produce alcohol from 1932 to 1937.

Table 10 gives the ethyl alcohol production of the United States, by kinds of materials used, for the fiscal year 1937.

Various types of raw materials are used in different countries. In Germany, for example, potatoes are used extensively; in France, sugar beets. Sweden manufactures much industrial alcohol from sulphite pulp. In Italy, sugar beets, molasses, grapes, and other substances are used.

PROCESSES OF MANUFACTURE

The process used in the manufacture of ethyl alcohol by fermentation depends on the nature of the raw material. Saccharine materials usually require little or no special preliminary treatment other than dilution and may be fermented directly after certain customary adjustments have been made in the mash. But starchy and cellulosic materials must be hydrolyzed to fermentable sugars before they can be utilized by the yeast.

In each of the processes, success depends on the efficiency of preliminary treatment, if any; the use of an optimum concentration of sugar, an optimum pH, and an optimum temperature; the addition of nutrient substances to the mash, if it be deficient in any essential constituent; inhibition of bacterial growth; the use of a vigorous strain of yeast with high alcoholic tolerance and so capable of producing large yields of alcohol; the maintenance of anaerobic conditions during the fermentation proper; and the prompt distillation of the fermented mash.

1. Ethyl Alcohol from Molasses. *a. Outline of Process.*—The molasses mash is adjusted to the desired sugar concentration and temperature by the addition of water and to the desired pH by the addition of a measured quantity of acid. A yeast "starter" is mixed with the mash in the fermentation tank, which is usually covered, in one of several manners. Streams of the adjusted mash and the starter flowing simultaneously into the fermenter may be caused to converge on a baffle board located in the upper part of the tank. The mash and starter become well mixed as they spatter and fall to the bottom of the tank. An alternate method is to add the starter after the mash has been placed in the tank and effect mixing of the two by compressed air from lines located at the bottom of the tank. Another method would be to use paddles.

The fermentation rapidly becomes vigorous with the evolution of large quantities of carbon dioxide. In the modern plant, this gas is

collected, purified, and used for the manufacture of dry ice or for other purposes. Within 50 hr., or less, the fermentation is usually complete. The fermented molasses, referred to as "beer," is distilled in a continuous still to separate the alcohol and other volatile constituents from the mash. The alcohol is purified by means of rectifying columns and then stored in a bonded warehouse or denatured.

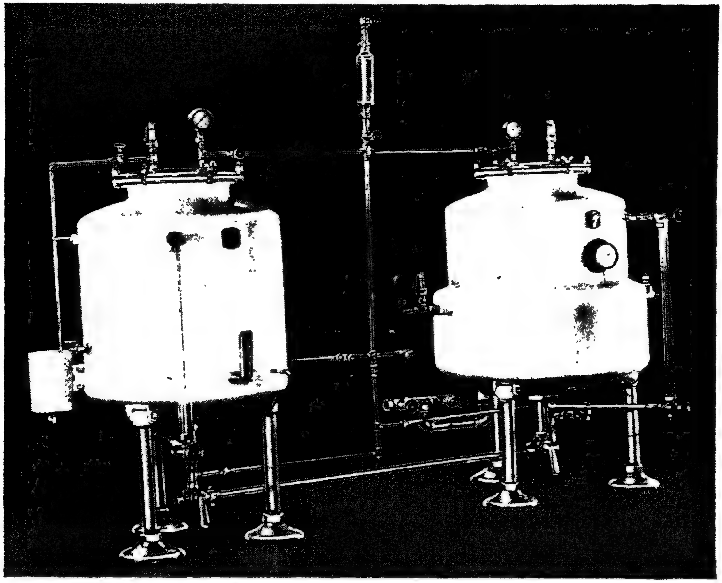
b. The Process in Detail. (1) TYPE OF YEAST.—Certain types of yeasts are desirable, namely, those which are able to produce and tolerate high concentrations of alcohol and which possess uniform and stable characteristics. Strains of *Saccharomyces cerevisiae* are commonly used, but other yeasts, such as *S. anamensis* and *Schizosaccharomyces pombe*, may be employed under certain conditions.

(2) PREPARATION OF STARTER.—Having selected the yeast for the fermentation and having isolated it in pure culture, a starter is then prepared. A starter of large volume is required to "pitch" (inoculate) the main mash, which frequently may have a magnitude of several thousand gallons. Using aseptic technique, a tube containing about 10 cc. of sterile wort is inoculated from a pure culture of the yeast, which may be maintained on malt agar media. After incubation for a suitable period of time at a temperature of 25°C. (77°F.) to 30°C. (86°F.), the optimum for yeast growth, the culture in the tube may be used to inoculate a flask containing approximately 200 cc. of sterile mash. Following incubation, the contents of the flask may be used to seed a sterile mash of about 4-liter capacity. Up to this point in the preparation of the starter, the work is ordinarily carried out in the laboratory, using glass containers. The next mash inoculated is of semi-plant-scale size (10 to 40 gal.) and is located close to the fermenters. Usually at least one more proportionally larger mash (several hundred gallons) is inoculated and permitted to incubate. Then this fermenting mash, the starter, is either pumped or allowed to flow by gravity to the main mash. The addition of this enormous yeast culture to the mash constitutes "pitching."

Aeration is of benefit in preparing a starter, for the object is to secure an immense number of yeast cells.

The Magné automatic system or other pure culture systems (Fig. 7) for preparing the starter may be used instead of the method just outlined. In the Magné apparatus a stock of the pure culture is maintained in the upper drum of the apparatus. Mashes are inoculated from this pure culture as required, and one culture may be used over a considerable period of time before recharging with a pure culture derived from a single cell. For further details, the reader may consult the reference given below.[1]

[1] J. H. P., U.S. Patent 1,212,656, Jan. 16, 1917.



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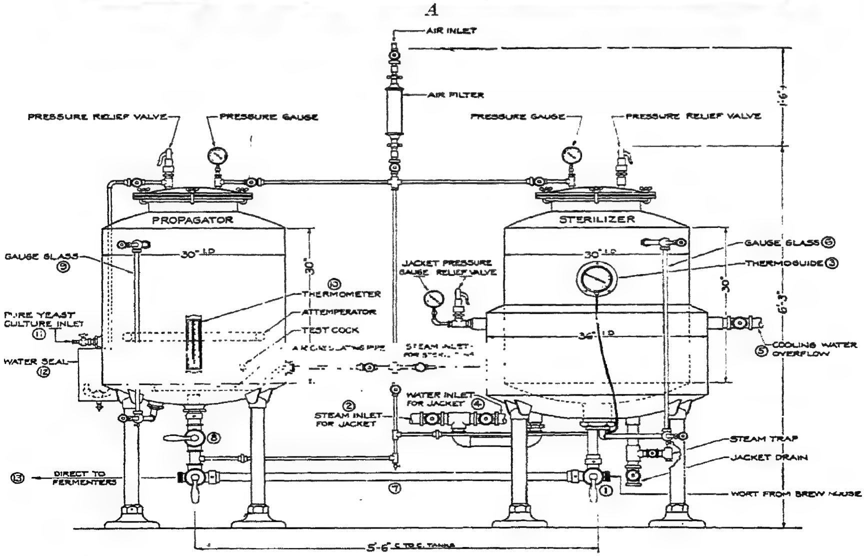

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Fig. 7.—Pure yeast propagator. (*Courtesy of the Pfaudler Co., New York City.*)

(3) THE MOLASSES.—"Blackstrap" molasses is the principal source of industrial alcohol. This material is the sirup that is left after the recovery of crystalline sugar from the concentrated juice of sugar cane. It usually contains 48 to 55 per cent of sugars, mainly sucrose. A large part of the blackstrap molasses used in this country is imported from Cuba, although some comes from Puerto Rico.

During recent years, much "high-test" molasses has been used for ethanol manufacture. This so-called "molasses" is an evaporated sugar-cane juice that contains all the original sugar of the juice, but most of it in an inverted form as a result of acid hydrolysis. Such molasses is usually high in sugar, containing occasionally as much as 78 per cent.

(4) CONCENTRATION OF SUGAR.—A sugar concentration of 10 to 18 per cent is usually satisfactory, although other concentrations are used. When the concentration is too high it reacts adversely on the yeast, or the alcohol produced may inhibit the action of the yeast, with the consequence that the fermentation time is prolonged and some of the sugar is not properly utilized. The use of too low a concentration of sugar is uneconomic as it may lead to a loss of valuable fermenting space. Furthermore a proportionally greater quantity of fermented wort must be distilled to produce the same amount of alcohol thus adding to the expense of production.

A sugar concentration of approximately 12 per cent is frequently used. The quantity of water required to reduce the molasses to the desired sugar content is calculated from available analytical data. For example, 4 parts of water by weight would be added to 1 part of molasses, containing 60 per cent sugar, by weight, to reduce the sugar concentration to approximately 12 per cent.

The concentration of the sugar in a wort is usually determined by means of a Balling hydrometer, an instrument that gives the approximate percentage of sugar at 60°F., or 15.5°C.

(5) NUTRIENT SUBSTANCES.—Although molasses generally contains most of the nutrient substances required for fermentation, ammonium salts, such as ammonium sulphate or phosphates, may be added to the mash to supply deficiencies in nitrogen or phosphorus.

(6) pH OF THE MASH.—Fermentation proceeds satisfactorily when the mash has been adjusted to a pH of 4.0 to 4.5. This pH favors the yeast but is sufficiently low to inhibit the development of many types of bacteria. The manufacturer depends on the pH of the wort and the use of a large inoculum to take the place of sterilization, since it is costly and impracticable to sterilize large molasses mashes. Ordinarily the main mash is inoculated with a starter that represents 4 to 6 per cent of its volume, although the starter may represent from 2 to 25 per cent of the volume of the main mash under certain circumstances.

Sulphuric acid is commonly used to adjust the reaction of the mash, although lactic acid is satisfactory. Lactic acid favors the development of yeasts but inhibits the growth of the butyric acid bacteria, which are detrimental to the yeast fermentation. As a substitute for the addition of acid, the mash may be inoculated with lactic acid bacteria, prior to the alcoholic fermentation.

(7) OXYGEN TENSION.—Oxygen in large amounts is necessary in the early stages for the optimum reproduction of yeast cells but is not required for the production of alcohol. During the fermentation, carbon dioxide is evolved and anaerobic conditions are soon established.

(8) TEMPERATURE.—The mash is pitched at a temperature of 60 to 80°F., usually 70 to 80°F., depending somewhat on the external temperature. During fermentation, the temperature of the mash rises. The use of cooling coils or sprays on the outside of the tank helps to maintain a suitable temperature. At temperatures much above 80°F., alcohol evaporates rather rapidly. Bacterial growth is also favored.

(9) TIME REQUIRED FOR FERMENTATION.—A fermentation is usually complete in 50 hr. or less, depending on the temperature, sugar concentration, and other factors.

(10) DISTILLATION.—The fermented mash ("beer") is distilled to separate the ethyl alcohol and fusel oil from the other constituents of the mash. In case there is a shortage of fermenters and the "beer" cannot all be distilled immediately, part of the "beer" is pumped to a storage tank, known as a "beer well," where it is held until it can be distilled.

During distillation, fractions containing different concentrations of alcohol ("wines") and slops are separated. The fractions containing approximately 60 to 90 per cent of ethyl alcohol are known as "high wines." These fractions are concentrated to 95 per cent ethanol by further distillation or fractionation. The fractions low in alcohol, the "low wines," are usually redistilled with new lots of "beer." The slops are ordinarily discarded but may be used in a number of ways. Sometimes the slops may be used as a substitute for some of the water in diluting molasses for a new mash. The solids from slops may be concentrated by heat treatment and sold as a fertilizer constituent, for they contain potassium and phosphates in addition to other components. The slops may be used as a core binder (in foundries) or as an adhesive for artificial stone (briquette).

(11) YIELD.—The common yield from blackstrap molasses amounts to approximately 90 per cent of the theoretical, on the basis of the fermentable sugars.

(12) COST OF PRODUCTION.—Jacobs and Newton[1] have shown that the probable cost for producing 95 per cent bulk ethyl alcohol in tanks

[1] *Op. cit.*

(the carbon dioxide and potash not being recovered) from molasses varies from 15 to 21 cts. per gallon, according to the data of Table 11.

TABLE 11.—COST PER GALLON OF PRODUCING ALCOHOL FROM MOLASSES

Item	Low	Average	High
Blackstrap molasses at $0.05 per gallon (2.3 to 2.7 gal. of raw material per gallon of alcohol)	$0.115	$0.125	).135
Conversion cost (labor, steam, chemicals, water and power, interest, depreciation, taxes, and insurance; credit deducted for fusel-oil by-product)	0.035	0.055	0.075
Cost of 95 per cent bulk alcohol in tanks	$0.150	$0.180	$0.210
Denaturing cost	0.020	0.025	0.030
Sales cost, freight, and profit	0.060	0.075	0.090
Total cost of 95 per cent denatured alcohol, unpackaged (tank cars)	$0.230	$0.280	$0.330
Additional cost, with molasses at $0.06 per gallon	0.023	0.025	0.027
Additional cost of producing "anhydrous" grade	0.02	0.025	0.030
Packaging	0.04	0.045	0.030

(13) FINAL TREATMENT.—The 190 proof ethyl alcohol (95 per cent by volume) may be further purified, dehydrated, or denatured as prescribed by the Bureau of Internal Revenue. Thus it is possible to purchase 95 per cent ethyl alcohol, with or without denaturant; c.p. (chemically pure) ethyl alcohol of 96 per cent concentration; absolute alcohol, U.S.P. (United States Pharmacopoeia); and anhydrous denatured ethyl alcohol (water-free alcohol).

(14) FLOW SHEET.—Schemes for the production of ethyl alcohol from potatoes, corn, and molasses are shown on page 49.

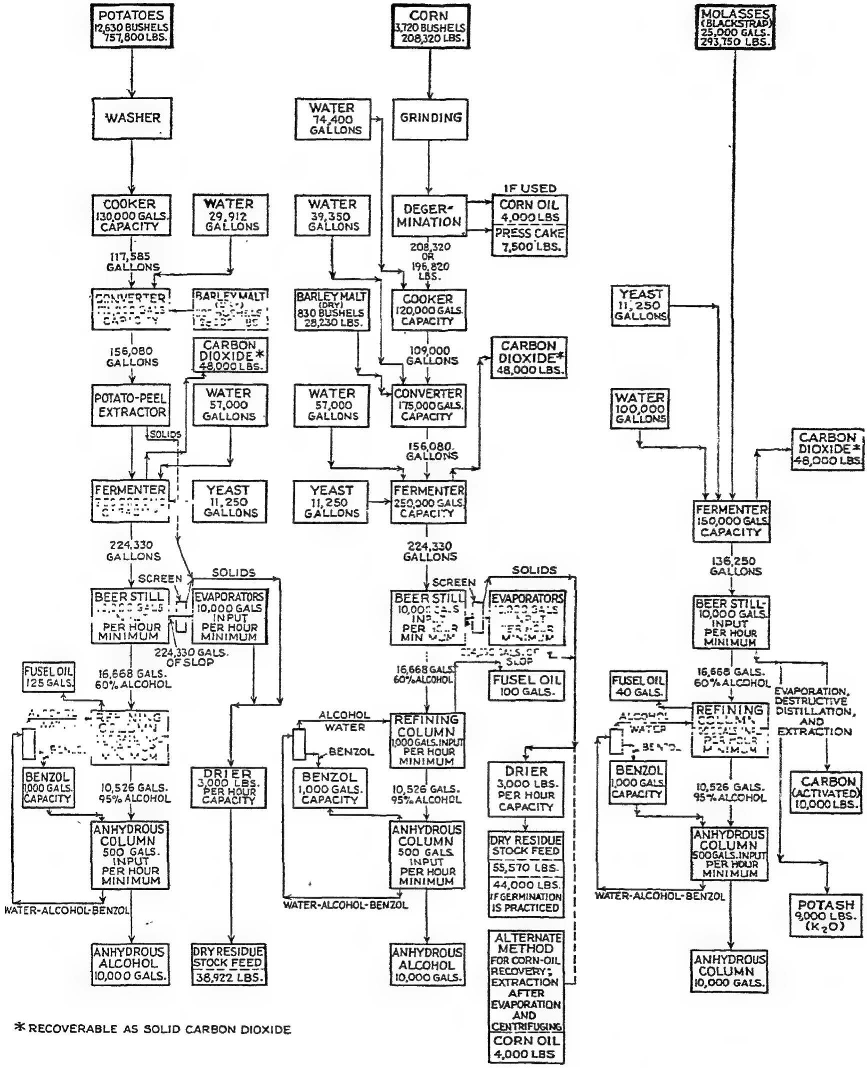
2. Ethyl Alcohol from Starchy Materials.—Since yeasts do not secrete amylases, it is necessary to saccharify starch by one of the methods cited in the following paragraph.

a. Methods of Hydrolyzing Starch.—There are two well-known methods for converting starch into fermentable sugars: enzyme hydrolysis and acid hydrolysis. Enzyme hydrolysis may be carried out by the use of malt, molds, or mold enzyme preparations.

(1) MALT HYDROLYSIS.—This method, which is frequently used, is considered in detail in the chapter on Brewing.

(2) THE AMYLO PROCESS.—This process is primarily one for converting starch to sugar by the use of selected molds, some of which have the ability to produce small quantities of alcohol from sugar.

The grain to be hydrolyzed is soaked in water for several hours to soften it. It is then mixed with approximately twice its weight of water



G. 8.—Conventional alcohol process. The residues, as shown, will be increased by the presence of yeast, yeast proteins, and unfermentable sugars. The figures actually used are based merely on the unfermentable residues calculated from the various analyses of the raw materials. (*Courtesy of P. Burke Jacobs and Harry P. Newton, Industrial Farm Products Research Division, Bureau of Chemistry and Soils, U.S. Dept. Agr., Misc. Pub.* 327, *December*, 1938.)

and heated at a pressure of 4 atmospheres to render the starch soluble. Acidification of the mash with 0.6 to 0.8 parts by weight[1] of hydrochloric or sulphuric acid per 100 parts of grain facilitates the liquefaction of the starch. The sterilized mash is cooled to 40 to 38°C. and inoculated with a pure culture of a *Mucor* or a related mold. *Mucor rouxii, Rhizopus japonicus, R. tonkinensis,* and *R. delemar* are some of the molds that have been used in the Amylo process. Sterile air is passed through the seeded mash for 24 hr., while the temperature is maintained at 38°C. The mash is then cooled to at least 33°C. and inoculated with yeast.

By using a modification of the Amylo process, developed by Boulard,[2] starch hydrolysis and fermentation are carried out simultaneously by adding at the same time *Mucor boulard* No. 5 and a yeast. It is claimed that time is saved by the use of the Boulard process.

(3) MOLD ENZYME PREPARATIONS.—Certain molds, especially strains of the *Aspergillus flavus-oryzae* group, are used in the manufacture of diastatic preparations. Takadiastase and *koji* are two such preparations, but these are not used on an industrial scale in this country for converting starches to fermentable sugars.

Underkofler and his associates[3] have demonstrated that higher alcohol yields, on the average, may be obtained from corn mashes saccharified by the use of moldy bran than from corn mashes saccharified by the use of dried barley malt. The experiments that led to these conclusions were carried out on a laboratory scale.

Preparation of the Moldy Bran.[3]—Approximately 30 g. of wheat bran is mixed with water in a 500-cc. Erlenmeyer flask to yield a mash of 50 to 70 per cent moisture content. The flask with its contents is then sterilized, using steam under pressure. The sterilized mash is cooled and inoculated with a well-sporulated culture of a selected strain of *Aspergillus oryzae*, incubated at 30°C., and agitated occasionally during the incubation period to prevent mats or lumps from forming. The well-sporulated bran culture thus prepared is used to inoculate a much larger charge (1,000 to 1,200 g.) of sterile bran of similar moisture content contained in a drum (holding about 5 gal.) capable of being rotated. During operation, air is passed slowly into the drum, which is maintained preferably at a temperature of about 30°C., and never above 35°C., to favor the growth of the mold. During the germination period, the drum is rotated for 15 to 20 min. or less each 2 hr. Thereafter the drum may be rotated continuously, at not more than 1 r.p.m. until, at the end of

[1] OWEN, W. L., The Amylo Process of Malting in Whiskey Distilleries, *Am. Wine Liquor Jour.*, June, 1936.

[2] BOULARD, H., Société d'exploitation des procédés H. Boulard, 1931.

[3] UNDERKOFLER, L. A., E. I. FULMER, and L. SCHOENE, *Ind. Eng. Chem.*, **31**: 734 (1939).

40 to 54 hr., the moldy bran is removed from the drum, spread thinly on a papered surface, and dried at room temperature. Sporulation proceeds during the drying. Before being used, the dried product is ground in the same manner as malt to saccharify corn mash.

The corn mash is saccharified by mixing ground moldy bran with it in the proportion of 8 to 10 lb. of bran per 100 lb. of corn and maintaining the mixture at a temperature of 45 to 60°C. for 60 min.

Moldy bran may be produced at a lower cost than barley malt, according to Underkofler and his coworkers.

Owing to the fact that an off-flavor or odor is produced in alcohol made from starch saccharified by the use of mold enzymes, this process would not be desirable for beverage alcohol, but could be used in the production of industrial alcohol for power purposes.

(4) ACID HYDROLYSIS.—The acid used for hydrolysis is usually sulphuric acid. The products to be converted are macerated, or ground, and mixed with a measured volume of water in acid-resistant equipment. A given quantity of acid is then added and the mass is hydrolyzed, using steam under pressure to facilitate the process. The mash may be neutralized by the addition of calcium carbonate or lime after the conversion is complete, the wort being separated from the precipitate of calcium sulphate by sedimentation and filtration. By extracting the precipitate with hot water an additional quantity of sugar may be recovered. Ammonia may also be used as a neutralizing agent, the ammonium sulphate formed acting as a source of nitrogen for the yeast.

In order to calculate the amount of calcium compound required to neutralize the mash, a measured portion of the mash may be titrated with standard sodium hydroxide to the desired pH, using the glass-electrode pH apparatus. An alternate method is to titrate a sample using a dye as the indicator, but in highly colored liquids it is usually somewhat difficult to adjust pH values accurately when using color indicators.

b. Ethyl Alcohol from Corn.—Nearly 200 million pounds of corn were used in the production of industrial alcohol during the Government fiscal year 1937.

(1) PRELIMINARY TREATMENT.—Corn with or without the germs is ground, mixed with water, and cooked with steam under pressure to gelatinize the starch. The mash is then blown to a converter tub where additional water is added. After the temperature of the mash has been reduced to about 140°F. (60°C.), barley malt is mixed with the mash. The enzymes, contained in the malt, convert much of the starch of the corn to a fermentable sugar (maltose) and also break down some of the protein material. (Refer to the chapter on Brewing for details of the action of malt.)

The mash containing the saccharified starch is transferred to a fermenter, adjusted to the proper sugar concentration by the addition of water, if necessary, cooled to a temperature of 65 to 80°F., and inoculated with yeast.

(2) FLOW SHEET.—A scheme for the production of ethanol from corn is shown in Fig. 8.

(3) COST.—It costs more to produce ethyl alcohol from corn than from molasses. It has been estimated[1] on the basis of the production of about 10,000 gal. of alcohol per day, that in 1933 it cost 31.4 cts. per gal. to produce anhydrous ethyl alcohol from corn costing 25 cts. per bushel; 43.5 cts. per gal., from corn costing 50 cts. per bushel; and 55.7 cts. per gal., from corn costing 75 cts. per bushel. Costs are less when the daily production is greater than 10,000 gal. per day.

(4) SOME SPECIAL USES.—Alcohol manufactured from corn is considered to be especially desirable for certain uses, on account of its freedom from foreign odors and flavors. For example, such alcohol may be used in the manufacture of perfumes, flavoring extracts, and high quality medicinals.

c. Alcohol from Jerusalem Artichokes.—The Jerusalem artichoke, or girasol (*Helianthus tuberosus*), native to North America, is a plant that has been studied in recent years as a possible source of levulose and/or industrial alcohol. Although this plant is cultivated on a large scale in some parts of Europe, it is not an important crop in the United States at present, except in a few localities.

The Jerusalem artichoke is rich in the polysaccharide inulin $(C_6H_{10}O_5)_n$, which is readily hydrolyzed to levulose.

(1) YIELD PER ACRE.—The Jerusalem artichoke has been studied by Boswell and his associates[2] in considerable detail. Investigating 20 varieties of the Jerusalem artichoke, all grown in three different parts of the United States for three different years, they found that the mean yield per acre was 6.58 tons at Urbana, Ill., 16.73 tons at Corvallis, Ore., and 8.74 tons at Washington, D.C. The mean yield of the 20 varieties at all three places for three years was 10.69 tons per acre.

(2) SUGAR CONTENT.—The sugar content of the tubers, after hydrolysis, varied in different seasons. A six-year mean analysis of the 20 varieties of Jerusalem artichokes investigated above showed 13.33 per cent levulose and 16.38 per cent total sugars.[2]

(3) STORAGE.—If an alcohol plant is to be operated continuously a constant supply of the raw material must be available. The fresh Jerusalem artichoke tuber cannot be stored satisfactorily. However,

[1] JACOBS and NEWTON, *op. cit.*

[2] BOSWELL, V. R., C. E. STEINBAUER, M. F. BABB, W. L. BURLISON, W. H. ALDERMAN, and H. A. SCHOTH, *U.S. Dept. Agr., Tech. Bull.* 514, May, 1936.

certain methods for storing the product have been worked out. McGlumphy and his associates[1] showed that the thinly sliced tuber could be satisfactorily desiccated and stored without sugar loss.

Both the dried and fresh tuber chips may be extracted by water in diffusion batteries, but such extracts deteriorate rapidly owing to microorganisms. If the extract, which contains the soluble sugars, is evaporated under reduced pressure to a total solid content of greater than 70 per cent, it becomes immune to decomposition by bacteria and yeasts. By storing the concentrated sirup under an atmosphere of carbon dioxide, mold growth is also prevented.[2]

The carbohydrates of the extract were stable at pH values between 4.8 and 9.0 at temperatures as high as 110°C.[2]

(4) FERMENTATION.—For fermentation, the sirup is diluted to yield approximately 12 per cent reducing sugars after hydrolysis, sterilized, cooled, and inoculated with yeast. *Saccharomyces cerevisiae*, *S. anamensis*, and especially *S. pombe* have been used with satisfactory results by Underkofler and his associates.[2]

It is not necessary to make a preliminary hydrolysis of the sirup or to add additional nutrient substances.

(5) CULTURE OF YEAST.—When the yeast was continuously cultured on unhydrolyzed sirup from artichokes, its ability to produce ethanol was increased.[2]

(6) YIELDS.—Yields of 90 per cent or more were obtained under the foregoing conditions.

3. Alcohol from Wood and Other Cellulose-containing Substances.—For many years scientists have been engaged in developing and improving methods and equipment for hydrolyzing wood and other cellulose-containing substances to sugars. Their efforts have been rewarded with success, for there are two processes, fundamentally different, by which sugar may now be prepared on a large scale from such materials. These processes are the Bergius process and the Scholler process.

Recently, an Italian process of rather high efficiency has been described.

a. Historical.—Dangiville, in 1880, proposed the use of gaseous hydrochloric acid to hydrolyze wood.

In Germany, Claassen[3] (1900) developed a method for hydrolyzing vegetable fibers, using sulphur dioxide or sulphuric acid. Attempts to

[1] McGLUMPHY, J. H., J. W. EICHINGER, R. M. HIXON, and J. H. BUCHANAN, *Ind. Eng. Chem.*, **23**: 1202 (1931).

[2] UNDERKOFLER, L. A., W. K. McPHERSON, and E. I. FULMER, *Ind. Eng. Chem.*, **29**: 1160 (1937).

[3] JACOBS and NEWTON, *op. cit.*

establish commercial processes in the United States, based on his methods, resulted in failure.

In 1910, Ewen and Tomlinson[1] constructed the first plant for producing ethyl alcohol from sawmill waste in Georgetown, S. C. The average yield of alcohol, using dilute sulphuric acid under high pressure to hydrolyze the sawdust, was approximately 20 gal. per ton, although higher yields were occasionally obtained.

Willstätter (1913) demonstrated that a 40 per cent solution of hydrochloric acid was very much different in action toward cellulose than the common 35 to 36 per cent commercial hydrochloric acid solution. Willstätter, with Zechmeister, discovered that cellulose is transformed without waste to glucose within the space of a few hours by a 40 per cent hydrochloric acid solution at room temperature.

TABLE 12.—PRODUCTION OF ETHANOL FROM CELLULOSE, BY DIFFERENT PROCESSES[1]

Process	Year	Hydrolytic agent	Concentration, per cent	Temperature, degrees Centigrade	Liters of ethanol per 100 kg. of dried wood
Braconnot	1819	H_2SO_4	100		
Arnould	1854	H_2SO_4	100		
Simonsen	1894	H_2SO_4	0.5	180	7.6
Claassen	1900	SO_2	.....	150	7.5
Ewen and Tomlinson	1909	SO_2	.....	150	8.0
Cohoe	1912	HCl and vapor	.....	140	9.0
Willstätter	1913	HCl	40	20	
Hägglund	1914	H_2SO_4	0.4	175	8.8
Kressmann	1915	H_2SO_4	2.5	174	10.7
Meunier	1922	H_2SO_4	.....	170	15–20
Scholler	1929	H_2SO_4	0.5	180	22–24
Bergius	1931	HCl	40	20	32–35
Giordani-Leone	1939	H_2SO_4	.....	...	30

[1] GIORDANI, M., *Chimica e Industria (Italy)* **21**: 265 (1939).

Hägglund, Bergius, and others commenced work in 1916 which led to the development of the Rheinau process.

During the World War, the Germans produced sugars from wood by the so-called "Stettin war process." This process corresponded to the process used by Ewen and Tomlinson in the United States. From August, 1918, to September, 1919, the plant at Stettin[2] produced 300 tons of sugar and converted it to 150,000 liters of ethyl alcohol. Owing to the low yields, only about 6 liters of alcohol per 100 kg. of dry wood

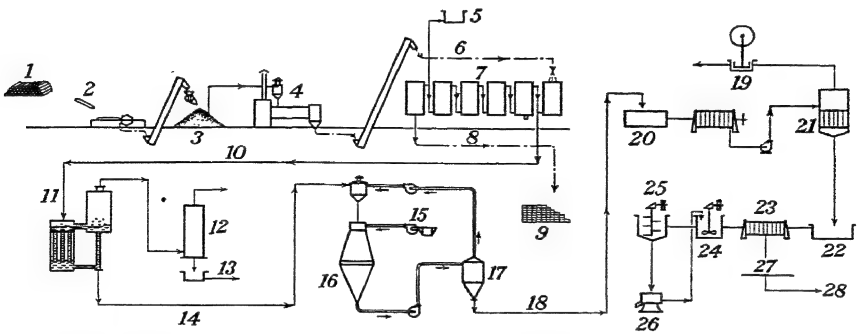
[1] JACOBS and NEWTON, *op. cit.*

[2] SCHOLLER, H., *Chem. Ztg.*, **60**: 293 (1936).

substance, it was economically unsound to continue operation of the plant after the war was over. It was later shown that the poor yields had been due to a partial destruction of the sugars during the hydrolytic process.

At Geneva, Terrisse and Lévy in 1920 used a combination of 40 per cent hydrochloric acid solution and gaseous hydrochloric acid to hydrolyze wood in a method that later became known as the Prodor process.[1]

Some research concerning the production of ethyl alcohol from wood wastes has been carried out by the U.S. Forest Products Laboratory at Madison, Wisconsin.[2]



1. Wood supply
2. Wood shredder
3. Chips
4. Wood dryer

Disintegration steps:

5. Hydrochloric acid
6. Dried wood chips
7. Diffusion battery
8. Lignin
9. Lignin-briquetting process

Evaporation steps:

10. Sugar solution
11. Evaporator for hydrochloric acid
12. Condenser
13. Hydrochloric acid
14. Sirup

Spraying steps:

15. Air heater
16. Spray dryer
17. Cyclone separator
18. Crude sugars (raw product)

Crystallization steps:

19. Vacuum pump
20. Inversion
21. Evaporator
22. First crystallization
23. Filter press
24. Solution vat
25. Recrystallization
26. Centrifuge for pure crystals
27. Sirup filtrate (mother liquor)
28. To alcohol (by fermentation)

FIG. 9.—Flow diagram of the Bergius process. (*Courtesy of Dr. F. Bergius, Ind. Eng. Chem.*, **29**: 247 (1937).)

Table 12, on page 54, summarizes some data concerning different methods for producing ethanol from cellulose.

b. The Bergius-Rheinau Process.—The Bergius[3] process is based on Willstätter's discovery that a 40 per cent solution of hydrochloric acid will hydrolyze cellulose to glucose at room temperature.

Cellulose is the principal constituent of wood. Hence a 40 per cent hydrochloric acid solution dissolves most of the wood except the lignin.

In the Bergius process, the wood is shredded and then dried in revolving drums to a water content of approximately 0.5 per cent. The dried

[1] ORMANDY, W. R., *Jour. Soc. Chem. Ind. (Trans.)*, **45**: 267T (1926).

[2] SHERRARD, E. C., *Chem. Age*, **29**: 76 (1921), etc.

[3] BERGIUS, F., *Ind. Eng. Chem.*, **29**: 247 (1937).

wood is next conveyed to a battery of diffusers, where hydrochloric acid acts upon it to convert it to water-soluble sugars and other end products. A countercurrent principle is employed in the extraction. The acid progresses through the battery (Fig. 9) and comes into contact with fresh wood in the first diffuser. During the extraction process, about two-thirds of the wood by weight is dissolved, while one-third remains as lignin. The resultant acid solution contains approximately 32 per cent by volume of reducing sugar.[1]

The acid solution containing the reducing sugars (the hydrolyzate) is now distilled at 36°C. under vacuum in order to separate the bulk of the acid from the sugar. This procedure removes about 80 per cent of the acid from the hydrolyzate. The acid is regenerated, reconcentrated, and used over again.

The hydrolyzate is further concentrated in a spray-drying chamber where water and hydrochloric acid are lost by evaporation. The resultant solid product, which is voluminous, is collected in a cyclone, an apparatus that removes particles of dried material from the air by centrifugal force.

There are approximately 90 per cent sugars, 8 per cent water, and 1 to 2 per cent of hydrochloric acid in the solid hydrolyzate.

The sugars of the hydrolyzate, which are usually glucose, fructose, mannose, galactose, and xylose (depending on the nature of the wood hydrolyzed), are principally in a tetrameric form and are not directly fermentable. However, by diluting the solid hydrolyzate with 3 parts by volume of water and by heating the resulting solution at 120°C. for 0.5 hr., the tetrameric form is converted to a fermentable form.[1] Galactose and xylose are not fermentable by yeast.

According to Bergius, approximately 80 per cent of the raw sugar obtained by hydrolysis is fermented to alcohol, a long ton of dry wood yielding 85 to 90 gal. of ethyl alcohol (190 proof). The other 20 per cent of the sugar may be recovered and used for fodder or for other purposes.

The lignin, washed free of acid, may be used as fuel, or pure charcoal may be prepared from it.

Acetic acid, in quantities that correspond to those recovered during the destructive distillation of wood, is obtained as a by-product of this process.

c. The Scholler-Tornesch Process.[2]—In contrast to the Bergius process, the Scholler process employs dilute acid, elevated temperatures, and steam under pressure. The wood used does not have to be dried or of any definite particle size.[2] Furthermore, no attempt is made to recover the acid.

[2] SCHOLLER, H., *Chem. Ztg.*, **60**: 293 (1936).

In 1926 and 1927 Scholler carried out research in the laboratory that indicated the value of the periodic removal of the sugar formed by pressure percolation. In Fig. 10 the curve X represents theoretical saccharification; the curve Z_1, the course of saccharification by the percolation method; and curve Z, the course of saccharification by a method that corresponds to the Stettin war process.

In the present industrial process, comminuted wood is carried by a system of conveyers to the top of a battery of percolators into which it is packed. Dilute acid solution is permitted to percolate intermittently

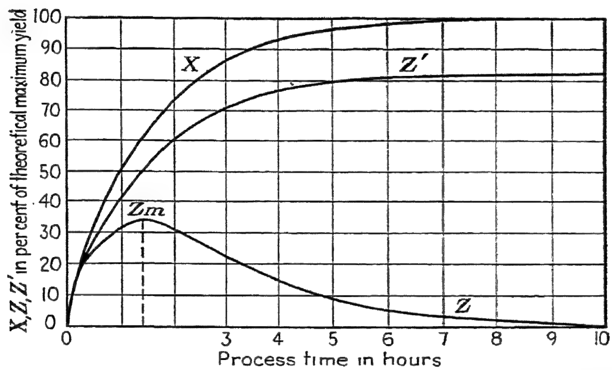


Fig. 10.—Graphical representation of course of saccharification in enclosed autoclave and percolator. (*Courtesy of Dr. H. Scholler, Chem. Ztg.*, **60**: 293 (1936).)

under pressure through the heated wood, the accumulating sugar solution being removed periodically to prevent it from becoming destroyed. The sugar solution is neutralized with $CaCO_3$, permitted to settle in a tank, and filtered. It may then be fermented by yeast.

As stated above, the particle size of the wood is not of great importance. Sawdust, plane dust, rasp dust, and similar types of wood of variable moisture content may be used directly.

Each percolator is 14 m. in length and 2.4 m. in diameter with a capacity for 50 cu. m. of material. It is filled loosely with the cellulose-containing substance. After the percolator is closed, steam pressure is applied suddenly from above upon the loosely packed material, with the result that it is compressed evenly. The percolator is filled again and the material again compressed. The procedure is repeated until the percolator is almost completely filled with the compressed cellulose material.

The dilute acid solution used for hydrolysis is prepared by pumping sulphuric acid into water, which has previously been warmed by passage through a heat-exchange apparatus, to give an acid concentration of approximately 0.4 per cent.

This dilute acid flows through the cellulose-containing material at a temperature of about 170°C. and a pressure of 8 atmospheres. The sugar solution thus formed is removed shortly, passing through the heat exchanger where it gives off heat to water being warmed for the dilute acid solution. Hydrolytic action continues in the percolator after the removal of the sugar solution, owing to the moisture, acid, heat (160 to 190°C.), and pressure, but no additional sugar is removed until the next passage of the hydrolyzing solution.

After the sugars have been recovered from the wood waste by intermittent percolation with dilute acid, the lignin residue, which is in the form of a hard cake, must be removed from the percolator. Since lignin has a high water content and is at a temperature of about 180°C. at the end of the process, the sudden release of a section in the lower portion of the percolator causes an explosive expansion of the water in the lignin and the cake is shattered.

From the sugars obtained by the preceding method, industrially important alcohols, acids, or yeast may be prepared.

Ethyl alcohol yields amounting to 60 to 70 per cent of the theoretical are obtained by this process.

Lignin, acetic acid, furfural, and waste sugars are by-products.

TABLE 13.—PRODUCTS OBTAINED BY DIFFERENT PROCESSES FROM 100 KILOGRAMS OF DRIED WOOD[1]

	Bergius, kilograms	Scholler, kilograms	Giordani-Leone, kilograms
Acetic acid.			0.2
Furfural....			2
	33	30	30
Total sugars..........................	66	43.3	60
Sugars fermented by yeast............	56	35.2	48
Sugars not fermentable by yeast[3]......	10	8.1	12
Yield of ethanol (liters of 100 per cent).	35	22	30

[1] Prepared from data presented by M. Giordani, *Chimica e industria* (*Italy*), **21**: 265-272 (1939).
[2] Data not available.
[3] Approximate figures. May be utilized by bacteria in production of butanol, etc.

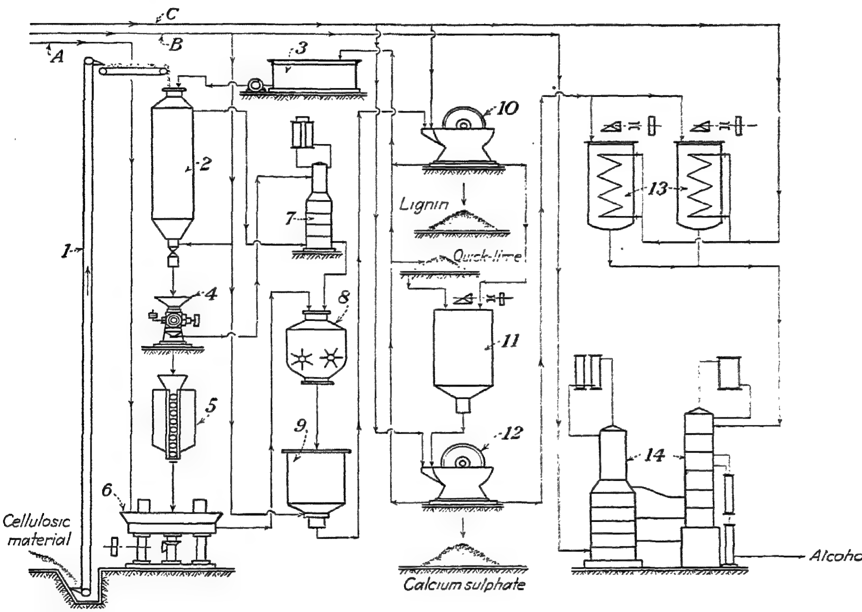
d. The Giordani-Leone Process.—This process, described and illustrated by Giordani,[1] makes use of 60°Bé. sulphuric acid. Space does not permit a detailed account of this process but some data concerning this, the Bergius, and the Scholler processes are presented in Table 13, on this page. Of the sugar derived from wood by the Giordani-Leone process, that part which is unfermentable by yeast may be used by *Clos-*

, M., *Chimica e industria* (*Italy*), **21**: 265–272 (1939).

tridium acetobutylicum for the production of butanol, acetone, and ethanol. The efficiency of the Giordani-Leone process is said to be high.

Figure 11 shows a scheme of the Giordani-Leone process for the saccharification of cellulosic materials.

e. Plants for Producing Ethanol from Wood.—The Societa Anonima Soterna is constructing plants for treating 30,000 tons of wood branches



1. Elevator
2. Autoclave
3. H_2SO_4(0.5 per cent) vat
4. Press
5. Dryer
6. Mash
7. Furfural column
8. Pulping apparatus
9. Hydrolysis vat
10. Lignin filter
11. Neutralization vat
12. Calcium sulphate filter
13. Fermentation tank
14. Distillation column

A. Pipe system of H_2SO_4 at 60° Bé.
B. Steam-pipe system
C. Water-pipe system

FIG. 11.—Scheme of Giordani-Leone process for the saccharification of cellulosic materials. (*Courtesy of Dr. M. Giordani, Chimica e industria (Italy)*, **21**: 265 (1939).)

annually.[1] The carbohydrates produced from the wood are to be used in the production of ethanol, glycerol, yeast, glucose, etc. Lignin, acetic acid, resin, and tannic acid will be by-products of the process.

4. Alcohol from Sulphite Liquor.—Sulphite liquor is produced as a waste product in the manufacture of pulp from wood.

[1] *Ind. Eng. Chem.* (*News Ed.*), **17**: 470 (1939).

a. Pulping Process.—Spruce, hemlock or some other kind of wood is cut into small chips that are subsequently digested with calcium bisulphite, using heat and pressure. The sulphite liquor reaches a temperature of 130 to 140°C. toward the end of the process. In some processes the temperature may rise even higher, with the result that some of the sugar in the liquor is destroyed. The cellulose pulp obtained by this method is used for the manufacture of paper.

b. Composition of Sulphite Liquor.—Together with each ton of pulp, there is produced 8 to 10 tons of sulphite liquor, which contains from 10 to 12 per cent of total solids. One analysis[1] of sulphite liquor showed the presence of the following products for each 1,000 kg. of cellulose obtained from Swedish spruce: lignin, 644 kg.; carbohydrates, 311 kg.; proteins, 15 kg.; resin and fats, 73 kg.; sulphur dioxide combined with lignin, 235 kg.; and calcium oxide combined with lignosulphonic acid, 102 kg.

The carbohydrates consisted of 49.4 per cent glucose, 15.6 per cent mannose, 8.1 per cent galactose, and 26.9 per cent of nonfermentable pentosans (arabinose).

Approximately 65 per cent of the total reducing sugars are usually fermentable.

c. Treatment of Sulphite Liquor.—Before the liquor is inoculated with yeast it is necessary to neutralize the sulphur dioxide, acetic acid, and formic acid that are present. Calcium carbonate or lime is commonly used for this purpose.

d. The Yeast.—In view of the nature of sulphite liquor, a special type of yeast is used to bring about fermentation of the sugars. Better yields are usually secured when the yeast is cultured in media containing sulphite liquor, for in this manner the yeast becomes acclimatized (compare with the glycerol fermentation).

e. Fermentation Period.—The fermentation is generally complete in 60 to 90 hr.[2]

f. Yields.—The yield of alcohol from sulphite liquors represents approximately 1 per cent of the volume of the medium.

g. Utilization of Sulphite Liquor.—During the Government fiscal year 1937, 12,060,125 gal. of sulphite liquor were used in the manufacture of industrial alcohol in the United States. The liquor was fermented in combination with molasses.[2]

Much industrial alcohol is produced from sulphite liquor in Sweden and other Scandinavian countries, also in Germany. Approximately 25,351,000 liters of 100 per cent ethanol were obtained from this source in Sweden during the year 1936.[2]

[1] EWESON, E. W., *Chem. Industries*, **38**: 573 (1936).

[2] JACOBS, P. B., and H. P. NEWTON, *U.S. Dept. Agr., Misc. Pub.* 327, December, 1938.

Sulphite liquor is used for the production of yeast on a large scale. Thus it is possible to obtain protein, vitamins, and other valuable con-

TABLE 14.—AVERAGE ANNUAL PRODUCTION OF CERTAIN AGRICULTURAL BY-PRODUCTS FOR 1931–1935[1]

By-product	Grain per bushel, pounds	Dry by-product per pound of grain, pounds	Dry by-product per bushel of grain, tons	Average production of dry by-product, 1,000 tons	Estimated quantity of dry by-product available for industrial use, 1,000 tons
Wheat straw	60	1.9	0.057	38,794	29,000
Rye straw	56	2.5	0.070	2,378	1,800
Oat straw	32	1.3	0.0208	20,156	0
Barley straw	48	1.2	0.0283	5,965	4,474
Flax straw	56	4.0	0.112	1,128	1,128
Rice straw	45	1.2	0.027	1,089	820
Total straws	..	....	....	69,510	37,222
Corncobs	56	0.22	0.00616	12,408	1,400
Oat hulls	32	0.30	0.0048	4,651	150
Rice hulls	45	0.20	0.0045	182	182
Cottonseed hulls	..	....	....	1,165*	583
	..	....	....	183†	92
Total cobs and hulls	..	....	....	18,589	2,407
Corn stover	56	1.2	0.0336	63,681	28,500
Cotton stems and pods	..	....	....	17,544‡	12,281
Bagasse fiber, continental United States	..	....	....	423§	423
Bagasse fiber, insular United States	..	....	....	3,276‖	3,276
Total other by-products	..	....	....	84,924	44,480
Grand total	..	....	....	173,023	84,109

[1] Senate Document 65, 76th Congress, 1st Session, "A Report of a Survey Made by the Department of Agriculture Relative to Four Regional Research Laboratories, One in Each Major Farm Producing Area," Washington, D.C., 1939.

* Reported production, but not limited to crop years specified.

† One-third of in-the-hull production.

‡ Calculated; 2:1 used as ratio of stems and pods to combined seed and lint.

§ Cane production ×0.125 for Louisiana and Florida.

‖ Cane production ×0.126 for Hawaii; ×0.12 for Puerto Rico and Philippine Islands.

stituents from a product that in many instances is still being dumped into streams.

Cellulosic Wastes.—Each year millions of tons of cellulose-containing materials are permitted to go to waste in the United States. In Table 14

are presented estimates concerning the production of various by-products, which may be termed agricultural wastes or residues. These data are, of course, subject to variation from year to year, since crop production is not stable.

TABLE 15.—AVERAGE YIELD[1] OF 99.5 PER CENT ALCOHOL PER TON[2]

Material	Gallons	Material	Gallons
Wheat (all varieties)	85.0	Yams	27.3
Corn	84.0	Potatoes	22.9
Buckwheat	83.4	Sugar beets	22.1
Raisins	81.4	Figs, fresh	21.0
Grain sorghum	79.5	Jerusalem artichokes	20.0
Rice, rough	79.5	Pineapples	15.6
Barley	79.2	Sugar cane	15.2
Dates, dry	79.0	Grapes (all varieties)	15.1
Rye	78.8	Apples	14.4
Prunes, dry	72.0	Apricots	13.6
Molasses, blackstrap	70.4	Pears	11.5
Sorghum cane	70.4	Peaches	11.5
Oats	63.6	Plums (nonprunes)	10.9
Figs, dry	59.0	Carrots	9.8
Sweet potatoes	34.2		

[1] Probable yield from a short ton of the raw material, calculated from the average fermentable content.
[2] JACOBS, P. B., and H. P. NEWTON, *U.S. Dept. Agr., Misc. Pub.* 327, December, 1938.

TABLE 15a.—AVERAGE YIELD[1] OF 99.5 PER CENT ALCOHOL PER ACRE[2]

Material	Gallons	Material	Gallons
Sugar cane (Hawaii, 18 to 22 months)	889.0	Pineapples	78.0
Sugar beets	287.0	Rice, rough	65.6
Sugar cane (Louisiana)	268.0	Pears	49.3
Jerusalem artichokes	180.0	Barley	47.9
Potatoes	178.0	Molasses, blackstrap	45.0
Sweet potatoes	141.0	Apricots	41.0
Apples	140.0	Oats	36.3
Dates, dry	126.0	Grain sorghum	35.5
Carrots	121.0	Buckwheat	34.2
Raisins	101.7	Wheat (all varieties)	33.0
Yams	94.0	Figs, fresh	31.5
Grapes (all varieties)	90.4	Figs, dry	29.5
Corn	88.8	Sorghum cane	26.4
Peaches	84.0	Rye	23.8
Prunes, dry	82.8	Plums (nonprunes)	21.8

[1] Probable yield calculated from the average fermentable content and based on present over-all average acre yield in the United States.
[2] JACOBS, P. B., and H. P. NEWTON, *U.S. Dept. Agr., Misc. Pub.* 327, December, 1938.

Yields from Various Raw Materials.—The probable average yield of 99.5 per cent ethyl alcohol per ton of raw material is shown in Table 15.

Table 15*a* supplies information concerning the probable average yield of ethyl alcohol per acre from various farm crops.

Government Supervision.—The purpose of Government supervision is to prevent the illegal use of untaxed alcohol. Ethyl alcohol is taxed, of course, to provide revenue for the Government. Should tax-free ethyl alcohol be used for beverage purposes, the tax from such alcohol would be lost.

Government supervision is carried out by Government officers who are stationed at all the manufacturing plants, the bonded warehouses, and the denaturing plants, none of which can be operated without permit from the Treasury Department. The officers keep records of the raw materials used, of the yields of ethyl alcohol, of the transfers of alcohol to the bonded warehouse, of transfers of alcohol from the bonded warehouse to the denaturing plant, and of the final disposal of the alcohol. The keys to the bonded warehouse are held by the Government officers. (For further details, consult Regulations 3, U.S. Department of the Treasury.)

Tax-paid Alcohol.—Tax-paid pure ethyl alcohol may be purchased without permit or bond for manufacturing purposes. State regulations must be complied with, however.

Tax-free Alcohol.—Alcohol " . . . may be withdrawn under regulations from any industrial plant or bonded warehouse tax free by the United States or any governmental agency thereof, or by the several States and Territories, or any municipal subdivision thereof, or by the District of Columbia, or for the use of any scientific university or college of learning, any laboratory for use exclusively in scientific research, or for use in any hospital or sanitarium."[1]

A person entitled to obtain tax-free alcohol, governmental agencies excepted, must first obtain a permit to purchase and then furnish bond as prescribed in Sections 3100–3124 of the Internal Revenue Code.

Denatured Alcohol.—Alcohol for use in many industries is denatured by bonded denaturing plants. "A denatured alcohol is ethyl alcohol to which has been added such denaturing materials as render the alcohol unfit for use as an intoxicating beverage."[2] Denatured alcohol may be completely denatured or specially denatured. A completely denatured alcohol is one that has been rendered totally unfit for beverage purposes by the addition of the substances specified in one of the formulas provided by the Bureau of Internal Revenue. Completely denatured

[1] U.S. Dept. of the Treasury, Bureau of Industrial Alcohol, Regulations 3, 1931.

[2] U.S. Dept. of the Treasury, Bureau of Internal Revenue, Appendix to Regulations 3, 1939.

alcohols may be used as antifreeze agents, for alcohol lamps, in insect powders, and for many other purposes. Such alcohol is available to manufacturers or the general public without obtaining a permit or filing a bond and, if sold and used according to the law and regulations, is not subject to the internal revenue tax.

Specially denatured alcohol is ethyl alcohol that has been rendered unfit for use as a beverage by the addition of one or more substances, but which is adapted for use in a large number of specialized industries or arts. Methyl alcohol (CH_3OH), benzol, pine tar (Pix liquida, U.S.P.), vinegar (containing not less than 9 per cent acetic acid), and ethyl ether are some of the denaturants which may be added to ethyl alcohol to render it specially denatured.[1] Specially denatured alcohol is "sold, possessed and used only pursuant to permit and bond, except as otherwise provided."[1]

According to the U.S. Treasury Department, there were 22,118,378 wine gallons of completely denatured alcohol and 80,084,281 wine gallons of specially denatured alcohol produced by the United States (including Hawaii and Puerto Rico) during the fiscal year 1937.

Table 16 shows some important uses of industrial alcohol.

Fusel Oil.—Fusel oil, which forms 0.1 to 0.7 per cent of the crude distilled spirit, is a mixture of amyl and isoamyl alcohols, principally; with smaller quantities of isobutyl and normal propyl alcohols and traces of acids, esters, and aldehydes. The following typical analysis[2] is given by Delbrück for the fusel oil obtained from the fermentation of potatoes: amyl alcohol, 68.76 per cent; isobutyl alcohol, 24.35 per cent; *n*-propyl alcohol, 6.85 per cent; and acids, esters, and furfural, 0.04 per cent. The composition and the quantity of fusel oil formed varies, however, according to the raw materials used and the nature of the fermentation. For example, 1 gal. of fusel oil may be obtained for each 1,000 gal. of ethanol in the fermentation of molasses,[2] but larger quantities may be obtained from potatoes and corn.

Fusel oil is used principally as a lacquer solvent. It is usually not refined or separated into its components.

A further discussion of some of the constituents of fusel oil will be found in the following chapter.

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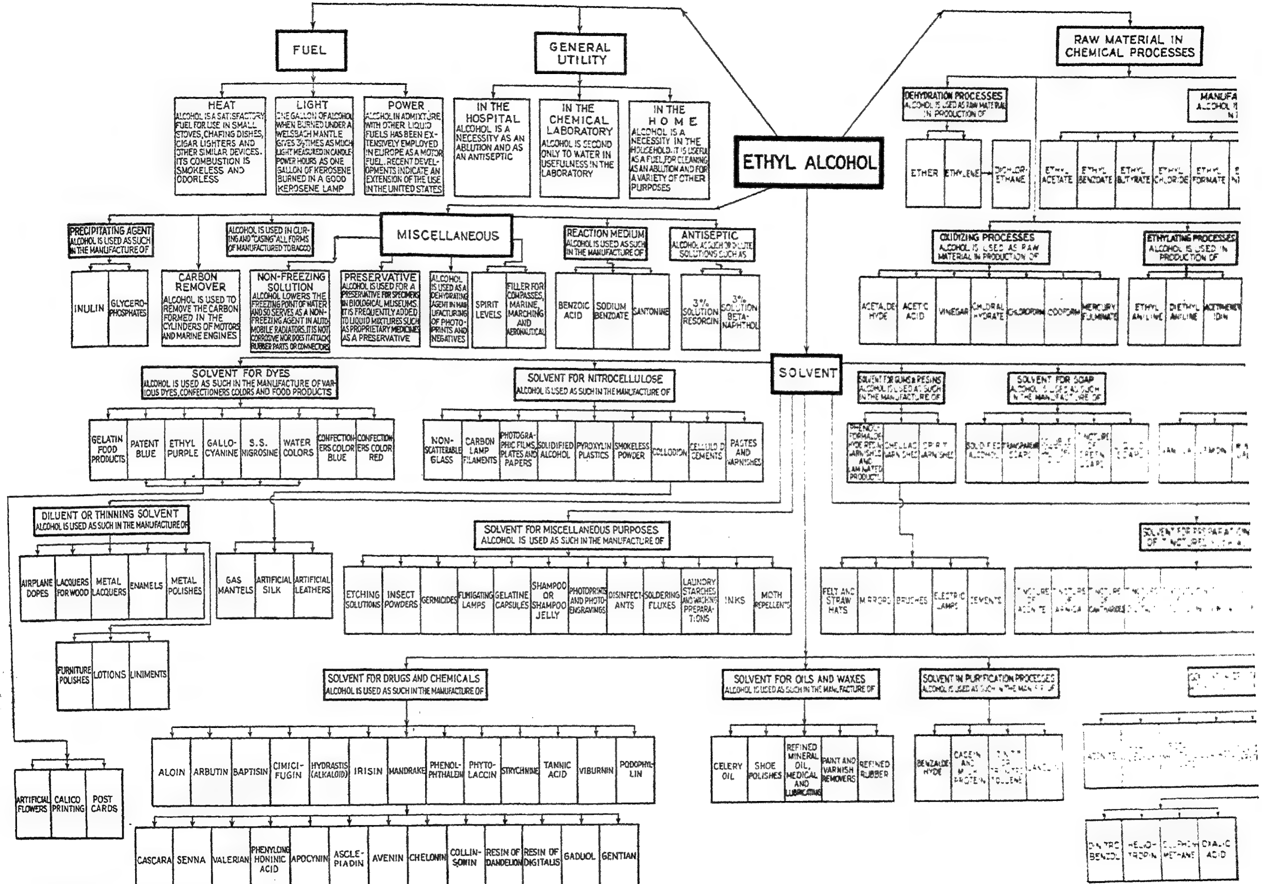
[2] Jacobs and Newton, *op. cit.*

TABLE 16.—IMPORTANT USES OF INDUSTRIAL ALCOHOL[1]



[1] Courtesy of the U.S. Industrial Alcohol Co.

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CHAPTER IV

FACTS AND THEORIES CONCERNING THE MECHANISM OF ETHYL ALCOHOL FERMENTATION

Some Early Researches.—In the earliest studies involving the mechanism of the ethyl alcohol fermentation, one was concerned only with the initial and end products. Black,[1] in the eighteenth century, stated that ethyl alcohol and carbon dioxide were the only products formed from sugar during the ethanol fermentation. Lavoisier, in 1789, carried out quantitative studies on this fermentation and found, besides ethyl alcohol and carbon dioxide, another product to which he gave the name "acetic acid." From 95.9 parts of sugar he obtained 57.7 per cent ethyl alcohol, 33.3 per cent CO_2 and 2.5 per cent acetic acid. Lavoisier's is believed to be the first quantitative work concerning the ethanol fermentation, although his results were incorrect.

Gay-Lussac investigated more intensively the field of research opened up by Lavoisier. He formulated the following equation to show what he believed was the essential chemical reaction of the ethanol fermentation:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} = \underset{\substack{\text{Ethyl}\\\text{alcohol}}}{2C_2H_5OH} + \underset{\substack{\text{Carbon}\\\text{dioxide}}}{2CO_2}$$

From 45 parts of glucose, he obtained 23 parts of alcohol and 22 parts of carbon dioxide.

By 1857, Pasteur had begun to publish the results of his researches on fermentation. He showed that 100 parts of sucrose gave rise to 105.4 parts of invert sugar, which in turn yielded 51.1 parts of ethyl alcohol, 49.4 parts of carbon dioxide, 3.2 parts of glycerol, 0.7 part of succinic acid, and 1 part of other substances. He thus proved that the weight relationships set forth by Gay-Lussac were in error.

Buchner,[2] in 1897, prepared a cell-free juice from yeast (by grinding the cells with sand and subjecting them to high pressure) which possessed the ability to produce ethyl alcohol and carbon dioxide from sugar. This discovery lent great impetus to subsequent studies concerning the intermediate reactions in the formation of ethyl alcohol.

Hexose Phosphates.—Harden and Young[3] (1905) discovered the importance of phosphates in cell-free fermentation. They have demon-

[1] Frey, C. N., *Ind. Eng. Chem.*, **22**: 1154 (1930).

[2] *Ber.*, **30**: 117, 1110 (1897).

[3] *Jour. Physiol.*, **32** (Proc., Nov. 12, 1904), 1905.

strated that inorganic phosphates disappear during the first part of the fermentation, while organic phosphates, *i.e.*, esters of hexose, are produced. They have described hexosediphosphate and proposed the following fundamental equation for fermentation by yeast extract:

$$2C_6H_{12}O_6 + 2PO_4HR_2 = 2C_2H_5OH + 2CO_2 + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$$

Hexosediphosphate is an important intermediate in the schemes of Embden, Meyerhof, and others for the breakdown of carbohydrates. This compound contains two phosphate, or phosphoric acid, molecules attached, one each, to the first and sixth carbon atoms of the hexose, which appears to be fructose. The same hexosediphosphate is obtained from glucose, fructose, or mannose.

At least two hexosemonophosphates, which have different properties, have been prepared: the Robison ester (glucopyranose-6-phosphoric ester), and the Neuberg ester (fructofuranose-6-phosphoric ester). When these esters are hydrolyzed, they yield glucose and fructose, respectively.[1]

Hexosemonophosphates have been synthesized by Levene and Raymond, Smythe, and others. A trehalosemonophosphate has been isolated by Robison and Morgan from a fermentation brought about by dried yeast.

Hexose phosphates are fermented at different rates. The diphosphate is fermented more slowly than glucose, while hexosemonophosphate is usually fermented more rapidly than the diphosphate, at a rate comparable with glucose in the initial stage only.[2]

The addition of hexose phosphates to cell-free yeast extracts containing glucose removes or greatly shortens the period of induction (the pause before fermentation starts). Without phosphates, no fermentation takes place.

The addition of phosphates to a medium does not affect the rate of fermentation by living yeasts.

For further details in respect to this very important subject, the reader is referred to Harden's monograph, "Alcoholic Fermentation," and to the bibliography that follows this chapter.

Methods of Studying the Mechanism of Fermentation.—Information concerning the mechanism of the ethyl alcohol fermentation may be gained by studying the related mechanism of lactic acid formation by muscle extracts; by the use of cell-free yeast juice or extracts, fixation methods, selective poisons, and dialysis; and by other means.

1. *Related Mechanism of Lactic Acid Formation by Muscle Extracts.*—Any productive advance in the study of the mechanism of lactic acid

R. A., "Outlines of Biochemistry," 2d ed., John Wiley & Sons, Inc., New York, 1938.

[2] Michaelis, L., *Ind. Eng. Chem.*, **27**: 1037 (1935).

formation by muscle extract has usually aided materially in the study of the mechanism of the ethyl alcohol fermentation, and vice versa. The researches of Embden on muscle extract exemplify the impetus that research on the former may do for the latter. The outstanding work of Meyerhof and his school add further proof to the valuable insight gained through correlative studies.

2. *Cell-free Extracts.*—Since the important reactions that take place in the conversion of sugar to ethyl alcohol occur normally within the living cell, since added hexose phosphates are fermented extremely slowly or not at all, and since the isolation of intermediates from the living cell is impossible in many cases, but comparatively little information concerning the intermediary reactions may be derived from studying such cells. Accordingly, cell-free extracts are used.

Two common methods of preparing the extract are the Buchner method (already mentioned) and the Lebedev[1] method. In the latter method the yeast is washed thoroughly with water, dried at 25 to 30°C., rubbed through a sieve, dried further, and stored until such time as an extract may be required. The extract is prepared by mixing 1 part by weight of dried yeast with 3 parts of water, incubating at 37°C. for 3 hr. and filtering the extract into a vessel cooled by ice.[2] It is important to use an extract that is free from living cells.

3. *Fixation Method.*—The fixation method was used with success by Neuberg. In this method a sulphite, such as calcium sulphite, or dimedon (dimethyl cyclohexane-dione), is used to fix the acetaldehyde as it is formed. The fixation product is not fermented by yeast and therefore accumulates in the medium. Since acetaldehyde is a hydrogen acceptor, the removal of this substance from the fermentation medium causes other hydrogen acceptors to become active. Use is made of this fact in the production of glycerol by fermentation, where the role of sulphites is very important.

Although pyruvic aldehyde forms an addition product with sulphites, this product is fermentable.

4. *Selective Poisons.*—Certain reactions in the normal chain of enzyme reactions do not take place in the presence of selective poisons, such as monoiodoacetic acid, sodium fluoride, and other substances. The poisons affect specific enzyme systems.

When monoiodoacetic acid is added to a cell-free extract containing hexosediphosphate, a mixture of triose phosphates forms as usual, which contains dihydroxyacetone phosphate and glyceraldehyde phosphate, but the reaction, in which triose phosphate is oxidized to phosphoglyceric

[1] LEBEDEV, A. VON., *Compt. rend.*, **152**: 49, 1129 (1911).

[2] STEPHENSON, M., "Bacterial Metabolism," 2d ed., Longmans, Green & Company, New York, 1939.

acid with the simultaneous reduction of acetaldehyde to ethyl alcohol, does not take place, owing to a poisoning of the enzyme system. Monoiodoacetic acid does not, however, retard the decomposition of phosphoglyceric acid.

Sodium fluoride, on the other hand, prevents phosphoglyceric acid from being broken down to pyruvic and phosphoric acids, but it does not prevent triose phosphate from being converted to phosphoglyceric and glycerophosphoric acids. Accordingly, phosphoglyceric acid accumulates in the medium.

5. *Dialysis.*—By means of dialysis, important constituents of yeast extract may be separated and studied.

The adenylic acid system and magnesium may be removed from yeast extract by dialysis. It is thus possible to study the reactions in which a gain or loss of phosphates is concerned.

Proteins, separated from coenzyme systems by dialysis, may be fractionated in the ways common to biochemistry.

6. *Other Means.*—There are other ways in which the intermediary reactions of fermentation may be studied.

Kluyver, Van der Lek, and others have demonstrated the value of studying the quantitative relationships of the intermediate and end products in formulating a scheme for the mechanism of fermentation.

Lohmann has obtained helpful information by determining the time required for normal hydrochloric acid to split off the phosphates from phosphorylated compounds.

Important information has been secured by studying the fermentability and fermentation rates of substances assumed to be intermediates.

A study of the formation and utilization of hexosemonophosphate has been made possible through the use of selected dyes. Naphtholsulphonate indophenol, brilliant alizarin blue or rosindulin GG inhibit the fermentation of glucose or of hexosemonophosphate but not of hexosediphosphate by yeast extract under aerobic conditions.[1] When hexosediphosphate is added to yeast extract containing glucose and one of these dyes, the hexosediphosphate is fermented. Contrary to expectations, the quantity of inorganic phosphate does not increase with the fermentation of hexosediphosphate, but it actually decreases, for an organic phosphate is synthesized. The reaction may be expressed as follows:

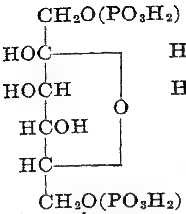
$$\cdot 2M_2HPO_4 + 4ROH \cdot \qquad \cdot 2CH_3CH_2OH + 2CO_2 + 2H_2O$$ *

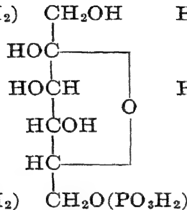
[1] Michaelis, L., and C. V. Smythe, *Jour. Biol. Chem.*, **113**: 717 (1936).

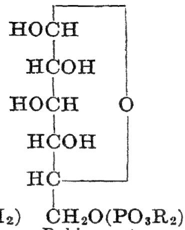
* Michaelis, L., V. Moraques-Gonzalez, and C. V. Smythe, *Enzymologia*, **3**: 242 (1937).

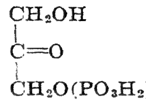
TABLE 17.—SOME IMPORTANT INTERMEDIATE AND END PRODUCTS OF THE ETHYL ALCOHOL FERMENTATION

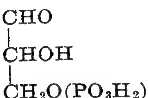
I. *Intermediate Products*

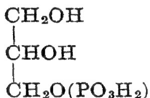

Fructo-furanose 1:6-diphosphate

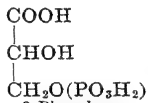

Neuberg ester

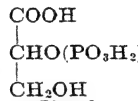

Robison ester

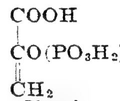

Dihydroxyacetone phosphate

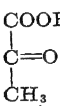

3-Glyceraldehyde phosphate


α-Glycerol phosphate

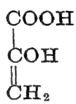

3-Phospho-glyceric acid

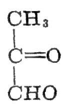

2-Phospho-glyceric acid

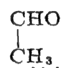

Phospho-pyruvic acid


Pyruvic acid

or

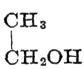

Pyruvic acid (enol form)


Methylglyoxal


Acetaldehyde

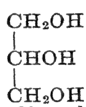
II. *End Products*

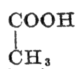
A. Derived from Sugar


Ethyl alcohol

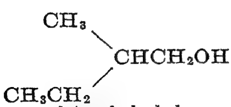
CO_2

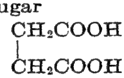
Carbon dioxide

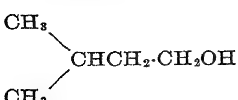

Glycerol


Acetic acid

B. Not Derived from Sugar


d-Amyl alcohol


Succinic acid

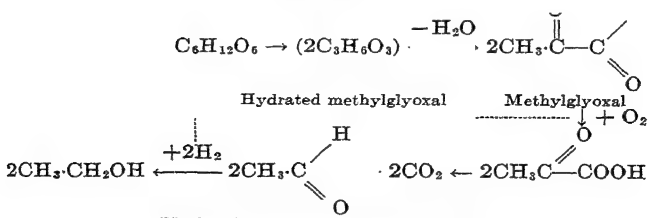

Isoamyl alcohol

The organic phosphate (RPO_4M_2) formed in the preceding equation appears to be hexosemonophosphate, a mixture of glucose, fructose, and mannose monophosphates.[1]

Precautions.—It must be borne in mind that the addition of substances to a medium, or yeast extract, may, and frequently does, alter the normal reactions. Furthermore the isolation of a substance that does not appear as an end product does not necessarily indicate that this substance is an intermediate in the main fermentation. Rigorous proofs, established by careful research, are necessary.

Neuberg's Scheme.—Neuberg's scheme[2] for the mechanism of the alcohol fermentation, based partly on the fact that pyruvic acid is an intermediate and is fermented at a rate equal to that of glucose and on the fact that a triose can be isolated, is as follows: 1 molecule of a hexose is broken down to 2 molecules of a triose ($C_3H_6O_3$), probably a hydrated form of methylglyoxal. The triose, through loss of water, forms methylglyoxal or an active form of this compound. Methylglyoxal, a ketonic aldehyde, is converted to pyruvic acid by oxidation, while another intermediate product is reduced. Pyruvic acid is decomposed to acetaldehyde and carbon dioxide under the influence of the enzyme, carboxylase. Acetaldehyde, in an acid medium, is reduced to ethyl alcohol, while methylglyoxal is being oxidized to pyruvic acid (a simultaneous oxidation-reduction). The scheme thus outlined may be illustrated as follows:

Scheme I



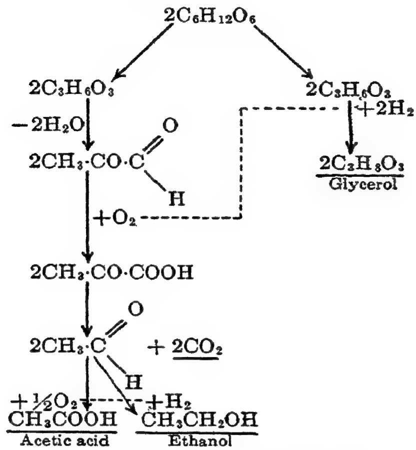
(Neuberg's "first form" of fermentation)

In case the reaction is alkaline, a compound, believed to be unstable and of the formula $C_3H_6O_3$, acts as a hydrogen acceptor together with acetaldehyde. The unstable $C_3H_6O_3$ compound is reduced to glycerol, while acetaldehyde is oxidized in part to acetic acid and reduced in part to ethanol. (A Cannizzaro reaction.)

C. V., *Jour. Biol. Chem.*, **118**: 619 (1937).

[2] Stephenson, *op. cit.*

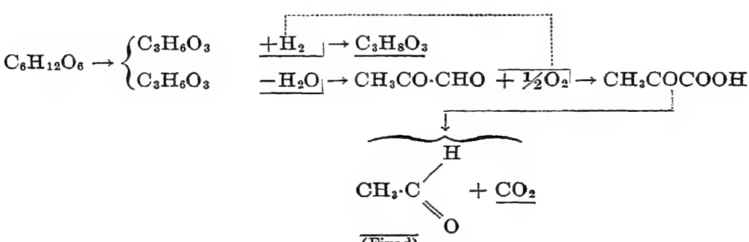
SCHEME II



(Neuberg's "third form" of fermentation)

One method of producing glycerol is by the sulphite fixation process. Acetaldehyde may be fixed by sulphite before it has become reduced or oxidized. The aldehyde thus fixed may be recovered from the fermentation medium and estimated. It is evident that any acetaldehyde that has become fixed is no longer available as a hydrogen acceptor. Acetaldehyde may, then, be replaced in part by $C_3H_6O_3$ as a hydrogen acceptor. $C_3H_6O_3$ is reduced to glycerol in proportion to the amount of acetaldehyde fixed (for each molecule of acetaldehyde fixed, 1 molecule of glycerol should be produced).

SCHEME III

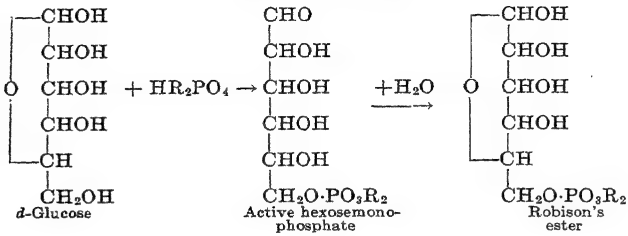


(Neuberg's "second form" of fermentation)

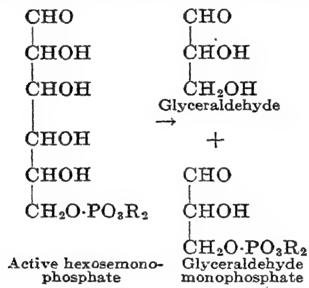
The normal fermentation, according to Neuberg, proceeds chiefly as shown in the first scheme, but some glycerol and acetic acid occur and hence the second scheme is realized in part.

KLUYVER'S SCHEME FOR THE ALCOHOLIC FERMENTATION OF GLUCOSE[1]

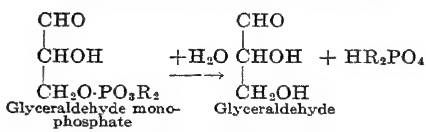
I. *Initial Phosphorylation*



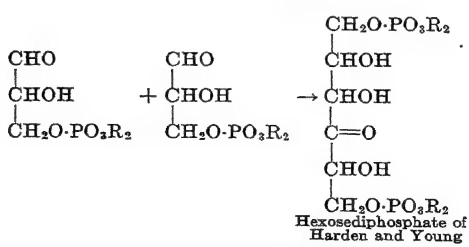
II. *Oxidoreduction of Hexosemonophosphate*



III. *Hydrolysis of Triosephosphate*



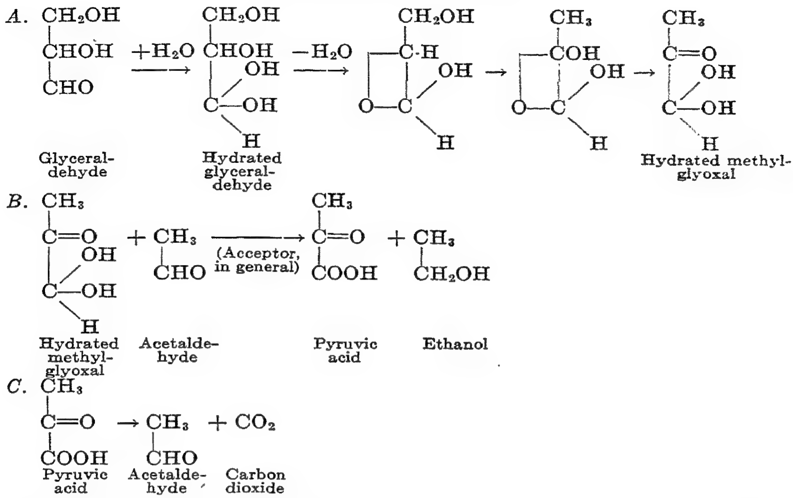
Condensation Reaction



[1] KLUYVER, A. J., Die bakteriellen Zuckervergärungen, *Ergeb. Enzymforsch.*, **4**: 230–273 (1935).

KLUYVER'S SCHEME FOR THE ALCOHOLIC FERMENTATION OF GLUCOSE.[1]—(*Continued*)

IV. *Final Oxidoreductions*



[1] KLUYVER, A. J., Die bakteriellen Zuckervergärungen, *Ergeb. Enzymforsch.*, **4**: 230–273 (1935).

Methylglyoxal has been isolated from the fermentation of hexosediphosphate by yeast extracts in water and toluol. It has likewise been isolated as a "semicarbazide" in the fermentation of sucrose by yeasts. It is not fermentable but may be regarded as the stable isomer of a fermentable form.[1]

An active hexosemonophosphate is first formed by the combination of glucose with a phosphate. Two 3-carbon compounds, glyceraldehyde and glyceraldehyde monophosphate, are the main end products formed through the splitting of the active hexosemonophosphate, but Robison's ester may be produced from this compound as a side product.

Glyceraldehyde monophosphate is hydrolyzed principally to glyceraldehyde and phosphate, while 2 molecules may condense by a side reaction to form the hexosediphosphate of Harden and Young. The phosphate liberated in the hydrolysis of glyceraldehyde monophosphate is available to react with more glucose to form more active hexosemonophosphate.

Glyceraldehyde is converted to hydrated methylglyoxal through a series of hydrogen transfer reactions. Methylglyoxal hydrate donates hydrogen to a hydrogen acceptor and is changed to pyruvic acid. Acetaldehyde acts as the hydrogen acceptor after it is once formed, being

[1] STEPHENSON, *op. cit.*

reduced to ethanol, while pyruvic acid is being produced. Pyruvic acid is decarboxylated to acetaldehyde and carbon dioxide. The acetaldehyde reacts with more methylglyoxal hydrate and the production of pyruvic acid, ethanol, acetaldehyde, and carbon dioxide continues.

Kluyver[1] believes that the catalytic agents responsible for the important changes in his scheme for the fermentation of glucose are an oxidoreductase and a phosphatase.

Scheme Based on the Researches of Meyerhof and Others.—In Fig. 12, a scheme for the intermediate reactions occurring in the ethyl alcohol fermentation is shown.

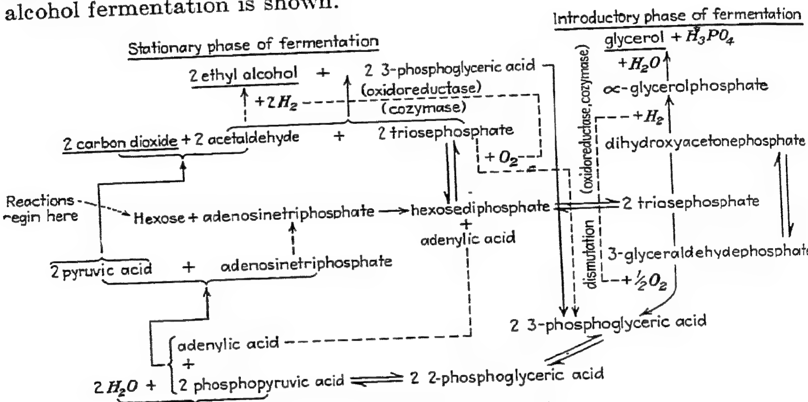
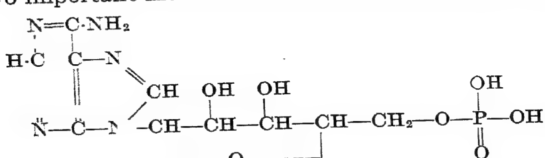


FIG. 12.—Scheme for fermentation.

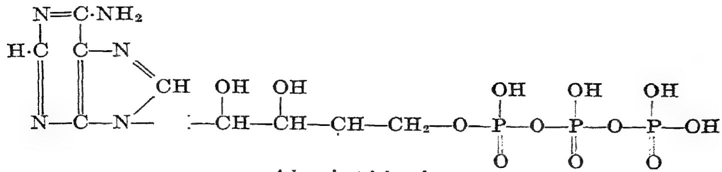
The first step, in the series of reactions, is a phosphorylation of the sugar (glucose, fructose, or mannose) in which a phosphorylating coenzyme, known as the adenylic system, and obtainable in a yeast dialyzate, functions.[2] This system, or coenzyme, consists of adenylic acid (adenosinemonophosphate), adenosinediphosphate and adenosinetriphosphate. The first two of these compounds have the ability to pick up phosphate, while the third, adenosinetriphosphate, donates phosphate. The formulas for two important members of the adenylic system follow:



Adenylic acid, or Adenosinemonophosphate

[1] For a further discussion of this subject, the reader is referred to A. J. Kluyver, "The Chemical Activity of Microorganisms," University of London Press, Ltd., 1931.

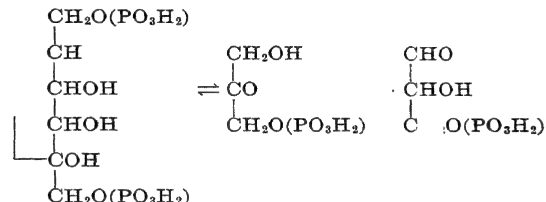
[2] MEYERHOF, O., *Nature*, **141**: 855 (1938).



Adenosinetriphosphate

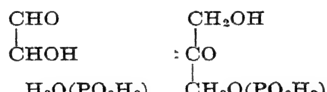
The hexose accepts phosphate from adenosinetriphosphate, the hexosemonophosphate appearing first,[1] then the hexosediphosphate upon further phosphorylation. Once the fermentation has reached the pyruvic acid stage, the phosphate given off by phosphopyruvic acid becomes available for phosphorylating additional hexose.

Fructose diphosphate, or diphosphoric acid (the common diphosphate obtained from glucose, fructose, or mannose), splits to form 2 molecules of triosephosphate with which it is in equilibrium:



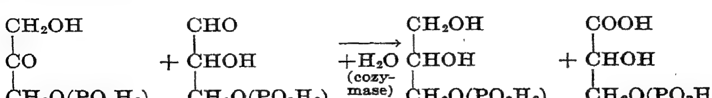
Fructose diphosphate Dihydroxyacetone phosphate 3-Glyceraldehyde phosphate

3-Glyceraldehyde is in equilibrium with dihydroxyacetone and is converted largely to the latter, according to Meyerhof.[1]



3-Glyceraldehyde phosphate Dihydroxyacetone phosphate

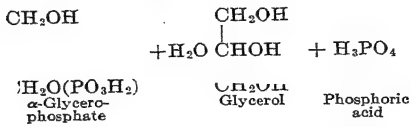
During the initial stages of fermentation (the induction period), before any acetaldehyde is produced, 2 molecules of triosephosphate dismutate to form 1 molecule of 3-phosphoglyceric acid and 1 molecule of α-glycerophosphate, this being an oxidation-reduction reaction in which cozymase (coenzyme I) is active:



Dihydroxyacetone phosphate 3-Glyceraldehyde phosphate α-Glycerophosphate 3-Phosphoglyceric acid

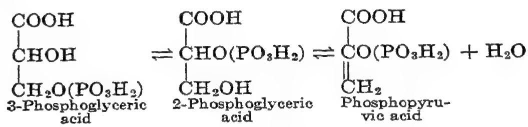
[1] *Ibid.*

α-Glycerophosphate is hydrolyzed to glycerol and phosphoric acid:



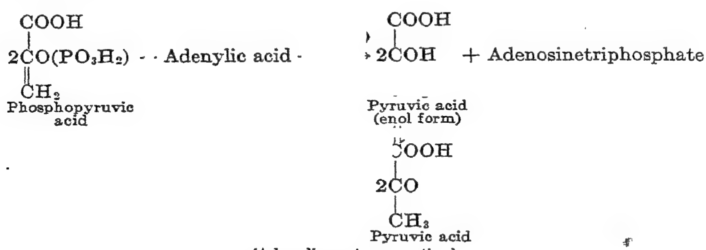
The preceding reaction accounts for the small amount of glycerol that is produced in the normal ethyl alcohol fermentation.

3-Phosphoglyceric acid proceeds, through a series of reversible enzyme reactions, to break down through 2-phosphoglyceric acid to phosphopyruvic acid:



The addition of sodium fluoride to a yeast extract containing either 3-phosphoglyceric acid or 2-phosphoglyceric acid prevents the formation of phosphopyruvic acid, for the enzyme (enolase) is poisoned.

Phosphopyruvic acid is dephosphorylated by adenylic acid to form pyruvic acid, the adenylic acid taking up phosphate to become adenosinetriphosphate.



(Adenylic system reaction)

Adenosinetriphosphate donates phosphate to 2 molecules of hexose to form 2 molecules of hexosemonophosphate and 1 molecule of adenylic acid, the net result being expressed by the following reaction:

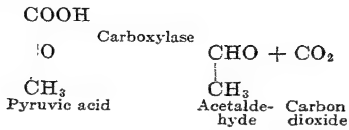
Phosphopyruvic acid + Hexose → Pyruvic acid + Hexosemonophosphate

Additional phosphopyruvic acid is broken down in the same manner with the result that the hexosemonophosphate is further phosphorylated:

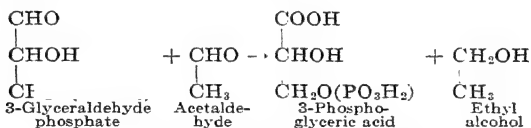
Phosphopyruvic acid + Hexosemonophosphate → Pyruvic acid + Hexosediphosphate

In this manner, hexosediphosphate arises during the stationary phase of fermentation.

Pyruvic acid is broken down to acetaldehyde and carbon dioxide by the enzyme carboxylase:



When present in sufficient quantity, acetaldehyde reacts with reduced cozymase (coenzyme I) with the result that acetaldehyde is reduced to ethyl alcohol while cozymase is oxidized. The oxidized cozymase now reacts with the triosephosphate (glyceraldehyde phosphate), oxidizing it to 3-phosphoglyceric acid, while cozymase becomes reduced again. It is thus seen that cozymase acts in the capacity of a hydrogen carrier in this oxidation-reduction. This reaction occurs normally during the stationary phase of fermentation.



(Cozymase oxidation-reduction reaction)

The phosphoglyceric acid thus formed is broken down through the series of reactions outlined above to produce more acetaldehyde, which reacts with more triosephosphate to produce more phosphoglyceric acid. The cycle continues in this manner to the end of the fermentation.

Types of Reactions.—Meyerhof[1] recognizes four main types of reactions in the foregoing scheme for the intermediate reactions of the ethyl alcohol fermentation. These include the phosphorylation-dephosphorylation reactions, the oxidation-reduction reactions, the reversible reactions, and the decarboxylation reaction.

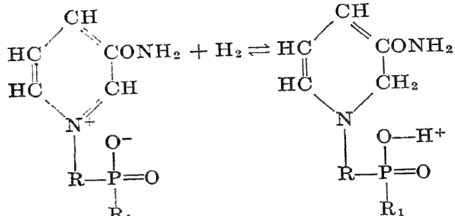
The adenylic system, the mechanism of which has already been explained, is concerned in the phosphorylation-dephosphorylation reactions.

Oxidoreductases and cozymase (coenzyme I) are responsible for the conversion of the triose phosphates to α-glycerophosphoric acid and 3-phosphoglyceric acid during the induction period and for the oxidation of triosephosphate (glyceraldehyde phosphate) with the simultaneous reduction of acetaldehyde during the stationary period of fermentation.

Nicotinic acid amide[1] is the hydrogen-carrying group of cozymase. The function of cozymase in transporting hydrogen may be illustrated

[1] *Ibid.*

by the following equation in which R represents the ribose phosphoric acid group of cozymase (see structural formula on p. 27) and R_1 represents the adenosinemonophosphate group:



Cozymase　　Reduced cozymase (Dihydrocozymase)

The principal reversible reactions include the reaction between hexosediphosphate and 2 molecules of triosephosphate; the reaction between dihydroxyacetone phosphate and glyceraldehyde phosphate; and the reactions between 3-phosphoglyceric acid, 2-phosphoglyceric acid, and phosphopyruvic acid.

A summary of the reversible reactions appears in the following scheme of Meyerhof.[1] The relation of each intermediate or end product to its immediate predecessor is indicated.

Glucose (Fructose, etc.)
↓ + $1H_3PO_4$
Glucose-6-phosphoric acid ⇌ Fructose-6-phosphoric acid
↓ + $1H_3PO_4$
Fructose (-1-6-) phosphoric acid
⇅

Dihydroxyacetone phosphate ⇌ 3-Glyceraldehyde phosphate
$-H_2$ ↑ ↓ $+H_2$ ↓ + O
α-Glycerolphosphoric acid 3-Phosphoglyceric acid
↓ ⇅
Glycerol + H_3PO_4 2-Phosphoglyceric acid
⇅
Phosphopyruvic acid + H_2O
↓
Pyruvic acid + H_3PO_4
↓
Acetaldehyde + CO_2
↓
Ethyl alcohol

Some Enzymes Active during Fermentation.—Several enzymes catalyze the intermediate reactions of the ethyl alcohol fermentation. Hexokinase converts fermentable hexoses to more active forms. Zymohexase catalyzes the breakdown of hexosediphosphate to triosephosphates. Oxidoreductase (mutase, dehydrase) with cozymase plays a very important role in the oxidation-reduction reactions. The conver-

[1] *Ibid.*

sion of phosphopyruvic acid to pyruvic and phosphoric acids is catalyzed by enolase, while carboxylase breaks down pyruvic acid to form acetaldehyde and carbon dioxide.

Basis for Theory.—Space permits only a relatively brief discussion of the facts upon which the foregoing theory is based. Many of the important discoveries were made during investigations of the intermediate reactions concerned in the formation of lactic acid in muscle extract and were subsequently established for the ethyl alcohol fermentation.

Parnas, Lutwak-Mann, and Mann have demonstrated the importance of adenylic acid and adenosinetriphosphate as dephosphorylating and phosphorylating agents, respectively.

Hexosediphosphate has been isolated repeatedly and is readily fermented by yeast and muscle extracts.

Hexosediphosphate enters into equilibrium with the 2 molecules of triosephosphate (Meyerhof and Lohmann, 1934): dihydroxyacetone phosphate, synthesized by Kiessling, and 3-glyceraldehyde phosphate, synthesized by H. O. L. Fischer (1932). These two triosephosphates have been isolated from muscle extract and subsequently from yeast extract fermentations of glucose or of hexosediphosphate with the aid of monoiodoacetic acid. They are readily fermented by yeast extract and together form an equilibrium in which dihydroxyacetone phosphate predominates. Whether one starts with a mixture of synthetic or natural triosephosphates or with hexosediphosphate a reversible reaction quickly occurs between hexosediphosphate and the triosephosphates in the presence of yeast extract.

Important information concerning the 3-carbon intermediates has been obtained by the use of sodium fluoride and monoiodoacetic acid. Embden (1933), using a muscle extract poisoned with sodium fluoride, showed that phosphoglyceric acid was an intermediate compound. He showed that in the absence of fluoride, phosphoglyceric acid was converted to pyruvic and phosphoric acids through enzymic action. Nilsson isolated phosphoglyceric acid from a fermentation by yeast extract.

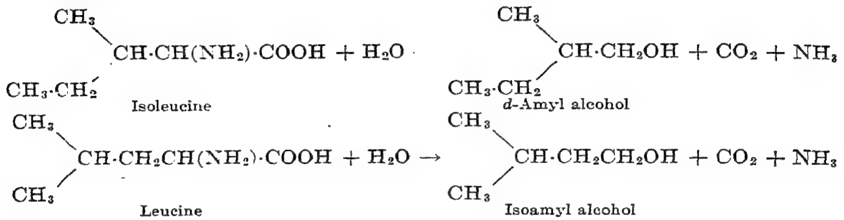
Meyerhof and Kiessling showed that there were two phosphoglyceric acids: 3-phosphoglyceric acid and 2-phosphoglyceric acid. These acids have been isolated from muscle extract and synthesized by Kiessling. They differ in structure, solubilities, and optical rotations.

Phosphopyruvic acid was isolated by K. Lohmann as a crystalline salt. It has been synthesized by Kiessling.

Pyruvic acid has been isolated as an intermediate and is readily fermented by living yeasts or yeast extracts. It is converted to acetaldehyde and carbon dioxide by the enzyme carboxylase, discovered by Neuberg.

Acetaldehyde has been demonstrated by fixation with sulphites and dimedon.

The Origin of Amyl and Isoamyl Alcohols.—Ehrlich[1] has shown that amyl and isoamyl alcohols are derived from the amino acids, namely, isoleucine and leucine, respectively. These acids are obtained from the medium usually, but in cases of nitrogen deficiency may originate from yeast protein.



The formation of these alcohols from their corresponding amino acids may be demonstrated by inoculating sterile mashes, containing a fermentable sugar and a measured quantity of one or both of the acids, with a pure culture of yeast and analyzing the mashes after the fermentation for the amino acids and alcohols. Control experiments should be carried out at the same time, of course, with mashes that do not contain added amino acids.

The ammonia set free in the foregoing reactions is utilized immediately by the yeast cells.

The relative proportions and likewise the quantities of the two alcohols formed depend on the composition of the medium, *i.e.*, on the relative amounts of the two amino acids, the presence or absence of ammonium salts, and the nature of the sugar; on the species of yeast; on the nutritive condition of the yeast; and on other factors. Certain ammonium salts cause a diminution in the normal yield of amyl alcohols, ammonia being derived from the salts rather than from the amino acids.

Leucine and isoleucine are not converted to their corresponding alcohols by living yeast cells in the absence of a fermentable sugar, nor are they converted by yeast juice or by zymin (a product prepared by treating yeast with alcohol and ether, or by acetone and ether).

Alcohols are produced from other alpha-amino acids in a similar manner by yeasts. Such products are believed to contribute to the flavor of beer, wine, rum, and other alcoholic beverages. For example, tyrosol, the alcohol produced from tyrosine, possesses a bitter taste and is concerned in the flavor of beer.

[1] Harden, A., "Alcoholic Fermentation," 4th ed., Longmans, Green & Company, New York, 1932.

Succinic Acid ($HOOC \cdot CH_2 \cdot CH_2 \cdot COOH$).—Succinic acid is believed to arise from glutamic acid during fermentation. Ehrlich discovered (1909) after experimentation with several amino acids, which included aspartic acid ($HOOC \cdot CH_2 \cdot CH(NH_2) \cdot COOH$), that glutamic acid was the only amino acid added to the fermentation medium that produced a well-defined increase in the quantity of succinic acid.

The probable course of its formation from glutamic acid is as follows:[1]

$$
\begin{array}{rl}
& HOOC \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH \quad \text{(Glutamic acid)} \\
& \downarrow \tfrac{1}{2}O_2 \quad \text{(Oxidative deamination)} \\
NH_3 + & HOOC \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH \quad \text{(α-Ketoglutaric acid)} \\
& \downarrow \quad \text{(Decarboxylation)} \\
CO_2 + & HOOC \cdot CH_2 \cdot CH_2 \cdot CHO \quad \text{(Succinic semialdehyde)} \\
& \downarrow \tfrac{1}{2}O_2 \quad \text{(Oxidation)} \\
& HOOC \cdot CH_2 \cdot CH_2 \cdot COOH \quad \text{(Succinic acid)}
\end{array}
$$

In the absence of added nitrogen-containing substances, succinic acid may be produced from the glutamic acid derived from the autolysis of the protein of yeast.

Succinic acid is not formed by yeast in the absence of sugar nor is it produced by yeast juice or zymin.

References on the Mechanism of the Ethyl Alcohol Fermentation

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CHAPTER V

BREWING

Brewing, or the production of malt beverages, is the name given to the combined processes of preparing beverages from infusions of grains that have undergone sprouting (malting), and the fermenting of the sugary solution by yeast, whereby a portion of the carbohydrate is changed to alcohol and carbon dioxide. It is an ancient industry and was probably invented by the Egyptians.

The term "malt beverage," according to the Federal Alcohol Administration Act[1] of May, 1937,

> . . . means a beverage made by the alcoholic fermentation of an infusion or decoction, or combination of both, in potable brewing water, of malted barley with hops, or their parts, or their products, and with or without other malted cereals, and with or without the addition of unmalted or prepared cereals, other carbohydrates or products prepared therefrom, and with or without the addition of carbon dioxide, and with or without other wholesome products suitable for human food consumption.

Beer, ale, porter, and stout are examples of malt beverages.

Composition of Beers.—The substances found in a beer will depend largely upon the nature and quality of the raw materials, the treatment of the sprouted grain or malt used in mashing, and the character of the ensuing fermentation, but storage and finishing operations will likewise affect the final composition.

In a normal beer one may expect to find carbohydrates—such as dextrin, maltose, and glucose—and protein derivatives such as peptones, amino acids, and amides, these products arising mainly as the result of the action of the enzymes of the malt. Hops contribute bitter substances, resins, essential oil, and tannic acid, but a portion of some of these substances is lost during the subsequent brewing operations. As a result of the alcoholic fermentation, the sugars of the wort are converted, in part at least, to ethanol and carbon dioxide, with much smaller quantities of glycerol and acetic acid; while some of the amino acids are transformed to higher alcohols and acids, for example, succinic acid. Lactic acid may be added to the wort or may accumulate as the result of the action of lactic acid bacteria. Salts are always found and usually

[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Federal Administration Act as in effect on May 15, 1937.

traces of oil. The finished beer contains 85 to 92 per cent of water by volume. Thus a beer is not a simple beverage but one that is capable of wide variation in composition unless the conditions of brewing are very carefully regulated.

Table 18 gives the analyses of several types of beer.

TABLE 18.—ANALYSES OF SOME MALT BEVERAGES[1]

Variety	No. of analyses	Specific gravity	Water	Carbonic acid	Alcohol by weight	Extract	Nitrogenous substances	Sugar as maltose	Gum and dextrin	Acid as lactic	Glycerin	Ash	Phosphoric acid
Schenk	205	1.0114	91.11	0.197	3.36	5.34	0.74	0.95	3.11	0.156	0.12	0.204	0.055
Lager	258	1.0162	90.08	0.196	3.93	5.79	0.71	0.88	3.73	0.151	0.165	0.228	0.077
Export beer	100	1.0176	89.01	0.209	4.40	6.38	0.74	1.20	3.47	0.161	0.154	0.247	0.074
Bock	84	1.0213	87.87	0.234	4.69	7.21	0.73	1.81	3.97	0.165	0.176	0.263	0.089
Weiss beer	26	1.0137	91.63	0.297	2.73	5.34	0.58	1.62	2.42	0.392	0.092	0.149	0.034
Porter	40	1.0191	88.49	0.215	4.70	6.59	0.65	2.62	3.08	0.281	.....	0.363	0.093
Ale	38	1.0141	89.42	0.201	4.75	5.65	0.61	1.07	1.81	0.278	.....	0.31	0.086

[1] Reprinted by permission from "Food Inspection and Analysis," 4th ed. by Leach and Winton, published by John Wiley & Sons, Inc., New York.

Extent of Manufacture.—Some idea of the magnitude of the industry may be gained by noting the following table, which gives the malt beverage production for the six leading states in the United States for the Government fiscal years 1936 and 1937. For each state, the number of breweries operated is also indicated.

TABLE 19.—MALT-BEVERAGE PRODUCTION, FISCAL YEARS 1936 AND 1937[1]

State	Production in barrels[2]		Breweries operated	
	1936	1937	1936	1937
New York	8,598,081	9,230,725	70	69
Pennsylvania	6,010,171	6,614,907	112	109
Wisconsin	5,736,534	6,448,420	91	86
Illinois	3,578,180	4,059,423	61	62
Ohio	3,432,921	4,281,448	54	55
Michigan	3,249,355	3,393,479	52	53
Total, United States	51,812,062	58,748,087	732*	720

[1] *U.S. Treas. Dept., Annual Report of Commissioner of Internal Revenue,* 1936 and 1937.
[2] The standard beer barrel holds 31 gal.
* Number operated during any part of the year.

During the fiscal year 1937, New Jersey, California, Minnesota, Indiana, Maryland, and Massachusetts followed the lead of the preceding states in the order named, while Missouri led Michigan by a small margin.

American Brewing Practice.—Beer produced in the United States is distinctive for two principal reasons. First, the American public prefers a sparkling clear, light, and very pale beer—one that will remain brilliant when very cold or warm. Second, owing to the differences in the composition and properties of our malts and cereals, departure from European procedures has become necessary.

The Brewing Process.—The essential steps in the manufacture of beer include the preparation of malt, mashing, boiling the wort with hops, fermentation, and finishing operations. Malt is prepared by soaking selected barley in water, permitting it to germinate and drying it under carefully regulated conditions. Malting is sometimes carried on as a separate industry, but it is so closely associated with the brewing industry that it may be regarded as an essential part of the whole brewing process. In mashing, ground malt is mixed with heated water and prepared malt adjuncts are usually added. The enzymes liberated by the malt digest the starches, proteins, and some of the other substances present in the mash. The soluble products are dissolved in the water of the mash and form the sweet wort. The mash is filtered, and the resultant clear wort is boiled with hops, strained, cooled, and pitched (inoculated) with selected yeast. A fermentation ensues, which is carried out at a low temperature. For lager beer the temperature is slightly lower than for ale. Following this fermentation, the beer is stored to mature or age. During the finishing operations, the beer is carbonated, cooled, racked, and pasteurized.

Although the principles employed in the production of beer and ale are essentially similar there are minor differences that are carefully regulated. A different treatment of malt is required both as to the length of the germinating period and the kilning of the malt; a higher temperature of fermentation is used, and a different type of yeast. Beer yeasts are so-called "bottom" yeasts, since the fermentation proceeds vigorously in the depths of the tanks and there is a great deposition of cells, while ale is fermented by "top" yeasts that produce great masses of cells in the foam that forms abundantly at the surface. Beer contains larger amounts of unfermented carbohydrates, especially dextrins, while ale has a higher percentage of alcohol. This is in part due to the more complete conversion of the starch to fermentable sugar in the malt used.

Malt.—Malt is an enzymic product prepared usually from selected barley, although other cereal grains may be used.

The Preparation of Malt.—The manufacture of malt, commonly carried out by maltsters, consists of steeping selected, screened barley, permitting it to germinate, and drying it under carefully regulated conditions.

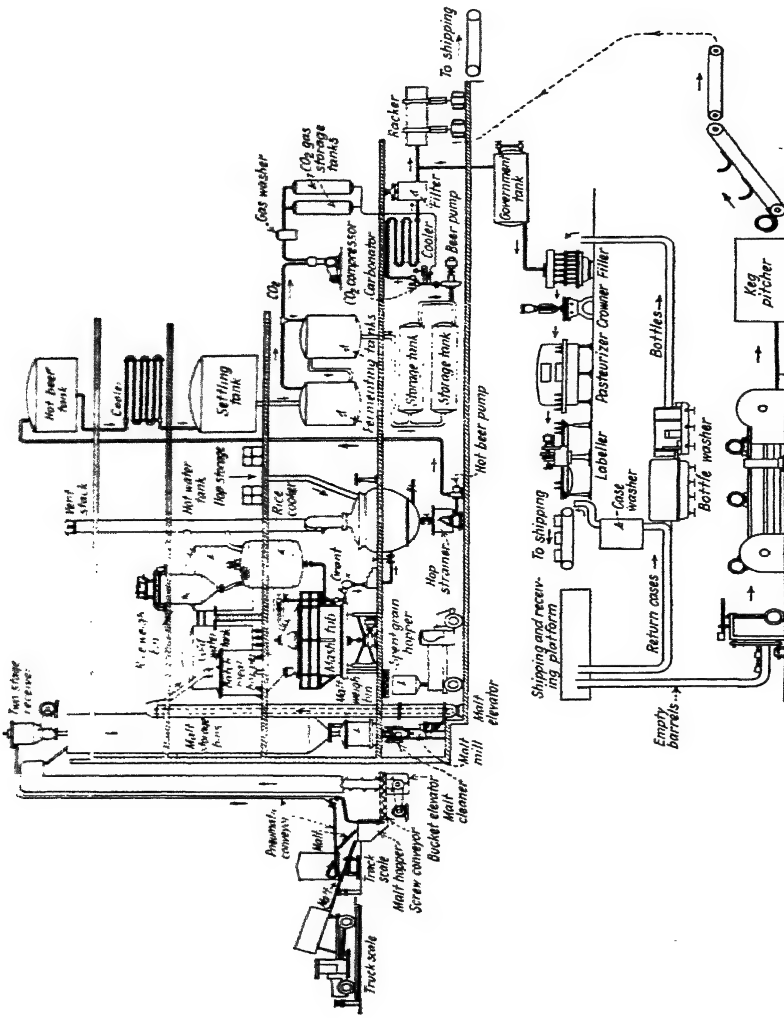

FIG. 13.—Brewery. (Courtesy of Dr. L. V. Burton, Editor, Food Industries.)

Selection of Barley.—Barley is selected for the results that it produces. It should be of a good variety, preferably large grained, of a fairly uniform size, and of a light yellow color when matured. It should possess high germinating power, contain but a small amount of bitter and harsh substances in the husk, and have the ability to produce enzymes of the proper character and quantity during germination. Soft, starchy barley is desirable; damaged or weedy barley is undesirable.

Certain types of barley are sought for the manufacture of fine beer. In the United States malt is prepared principally from six-rowed barley, although some two-rowed barley is used in California and much of it in Europe.

Composition of Barley.—Barley contains the four plant proteins: glutelin, hordein, leucosin and edestin. Glutelin and hordein are found mainly in the husk and aleurone cell layers; leucosin and edestin in the endosperm between the starch granules.

Starch is found in the endosperm and is a most important constituent. Fat, rich in lecithin, is found in the embryo and in the aleurone cells. Tannins and bitter resins are located principally in the husks. Together with the protein, the tannins and bitter resins are collectively referred to as "testinic acid."

Steeping.—Steeping is a process of soaking the grain in order that it may take up sufficient moisture to quicken the living cells of the embryo and start the processes of enzyme production and germination, which precede the breakdown of cell walls and the hydrolysis of the stored foods.

Before the barley is steeped it should be screened or sorted in order to obtain grains of a uniform size. The moisture content can then be controlled more readily during steeping.

During steeping, water is imbibed by the individual grains until an optimum moisture content of 45 to 47 per cent is obtained. This concentration of water favors normal germination.

Water of a known chemical composition and of a definite pH should be used during the steeping process. The composition of the water has much to do with the proper removal of the tannin, bitter resin, and some proteins, which are undesirable for beers. Since these substances dissolve more readily at a higher pH, the steeping water is sometimes made alkaline by the use of lime. The use of alkaline steep water apparently is of advantage when malt, and subsequently beer, is made from coarse barleys, but of no advantage when fine-skinned barleys are used.

Hypochlorites and permanganates have been used to some extent in the steep water. It is claimed that they may stimulate germination and destroy harmful microorganisms in the water. The value of their use is not firmly established, however.

The rate at which the water is absorbed by the grains depends on the variety of the barley, the size of the grain, and the temperature of the water mainly.

A temperature of 50 to 60°F. in the steeping tanks is satisfactory. Control of the temperature is very important.

Malting is essentially a vital process involving growth and respiration. Respiration rates increase with a rise in temperature and with augmented water content, and oxygen is naturally required. Therefore aeration of the steep water is essential. Aeration also causes foreign material and small defective or light barley grains to rise to the surface of the steeping tank where they may be removed by skimming. As an alternate method of aeration, the water may be drained off and the tank refilled.

Understeeping results in a reduced rate of respiration, abnormal growth of the rootlets and an incomplete breakdown of the proteins. Less extract can be secured from the final product.

Oversteeping leads to the production of a higher percentage of ungerminated grains, caused by a deficiency in the oxygen supply during steeping, the inadequacy being due to the increased rate of respiration. If the water is adequately aerated, the grains will germinate subsequently, but the development is likely to be abnormal. Hence oversteeping may lead to low yields of malt.

Germination.—During germination several complex changes take place. The visible morphological changes include the formation of the acrospire, or plumule, and rootlets. A histological examination would show the disappearance of the cell walls of the endosperm, while a biochemical analysis would indicate that certain metabolic changes had taken place—the breakdown of proteins, starches, and other complex constituents under the influence of enzymes. Enzymes are elaborated or activated when the temperature, moisture, and aeration conditions are satisfactory for the germination of the seed.

The temperature, moisture content of the grains, and oxygen supply are very important during germination. These are controlled very carefully in pneumatic chambers or slowly revolving drums. Helicoidal rakes keep the germinating grains continually turned over in the pneumatic chambers. In the drums, air is supplied through tubes, and the humidity is carefully controlled.

The temperature range of 68 to 77°F. (20 to 25°C.) is considered to be optimum for germination. At lower temperatures the process is slower.

The oxygen supply is especially important during germination, for the rate of respiration increases greatly in germinating grain. During respiration, heat energy is evolved, while carbon dioxide and water are

produced as waste respiratory products. The accumulation of carbon dioxide inhibits normal respiration, and its concentration or a deficiency of oxygen, or both, induces abnormal respiration in which hydrogen acceptors other than oxygen may function. The products formed during abnormal respiration may include acids, alcohols, and aldehydes, which exert a toxic effect on the germinating grain or young plant. Germinating barley usually shows a respiratory coefficient of approximately 1.

$$\text{Respiratory coefficient} = \frac{\text{Volume } CO_2 \text{ evolved}}{\text{Volume } O_2 \text{ absorbed}}$$

In the case of abnormal respiration, the respiratory coefficient increases, for the carbon dioxide increases, the oxygen decreases, or both changes occur simultaneously. For these reasons it is most important to secure good carbon dioxide removal and to turn and properly aerate germinating barley.

Drying the Germinated Barley.—When the barley has reached the desired stage of growth, drying stops the germination processes and with the application of heat develops color, flavor, and aroma and reduces the moisture content of the malt. A final moisture content of approximately 5 per cent is sought. Malt may then be stored without danger of breakdown by microorganisms.

Drying is carried out in thermostatically controlled kilns or drums at carefully regulated temperatures. The temperatures used have much to do with the enzyme content of the final malt and the flavor of beers made from it. Since much damage can be done to germinated barley by the application of too high temperatures when the grain is wet, low temperatures must be used at first. The temperature is raised gradually, or elevated by small increments, until a final kilning temperature of 75 to 100°C. is used.

The final temperature used depends upon the nature of the malt desired. For a light malt, a lower temperature should be used; for a dark malt, a higher temperature.

During recent years, some malts have been dried in vacuum drums. Such malts possess a high concentration of enzymes but, though satisfactory for use in distilleries, are not so suitable for brewing, since the usual protein changes are affected.

Following the kilning, the malt may be treated, usually by friction, to remove the radicles.

Enzymes.—Malted barley contains carbohydrases, proteases, lipases, phosphatases, and desmolases.

The starch molecule is dephosphorylated by the enzyme amylophosphatase.[1] Phosphate is liberated and the viscosity of the starch paste is

[1] Waldschmidt-Leitz, E., und K. Mayer, *Zeit. physiol. Chem.*, **236**: 168 (1935).

reduced. It is believed that amylophosphatase may also attack linkages other than phosphate linkages. The ability to produce liquefaction is, however, one of the principal functions of amylophosphatase.

Alpha-amylase and beta-amylase, diastatic enzymes dextrinize and saccharify starch after it has been liquefied. α-Amylase acts principally to dextrinize starch, while β-amylase saccharifies starch or dextrins.

According to Hanes,[1] α-amylase ruptures the 30-glucose starch macromolecule to yield dextrins, there being 3 to 17 glucose molecules in a dextrin. β-Amylase may attack the freed dextrins or the nonaldehydic end of the starch macromolecule and split off molecules of maltose. About 60 per cent of the starch is thus converted by β-amylase to maltose. The remaining unconverted portion is known as α-amylodextrin or erythrogranulose.

During germination, a proteinase brings about a breakdown in the proteins before diastatic activity is developed to any appreciable extent. Protein molecules are degraded to soluble products, among them peptides. These are further attacked by peptidases, of which there are at least two in germinating grain, and broken down to amino acids.

Several polysaccharides are found in the structure of the cell walls, for example, cellulose, xylan, pectin, and mannan. These are attacked by enzymes known as cytases—cellulase, xylanase, pectinase, and mannase, respectively.

Structure of Starch.—The starch molecule, according to Hanes,[2] consists of 30 glucose molecules in a straight chain, the molecules being linked by a series of oxygen bridges formed by the splitting of water from the free hydroxyl groups of the first and fourth carbon atoms. The starch molecule may be regarded as constructed of 15 maltose molecules. The 30-glucose molecule is referred to as a "starch macromolecule" and constitutes soluble starch.

Natural starches may have molecular weights of 20,000 to 200,000. It is believed that the 30-glucose units may be linked by phosphate and other linkages to make up the large natural molecule. The breakdown of the large molecule is accompanied by a decrease in viscosity and constitutes liquef

The straight chains are bound as a colloidal complex by phosphate. Very small amounts of fatty acids and nitrogenous matter (0.1 to 0.3 per cent) are believed to be a definite part of the starch molecule.

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[1] HANES, C. S.: *New Phytologist*, **36**: 101 (No. 2), 189 (No. 3) (1937).
[2] *Ibid.*

pean countries. A high nitrogen content is usually undesirable for it tends to produce a satiating and relatively unstable beer.

Besides reducing the nitrogen content of the wort, malt adjuncts supply additional carbohydrate to be acted on by the excess of diastase present and to be fermented by the yeast, and they help to produce a beer that is less satiating, paler in color, and relatively more stable.

Malt adjuncts include rice and corn products, cooked or uncooked. Corn grits, corn flakes, corn sugar (glucose), and corn sirups; dextrin; wheat flakes; sucrose; and invert sugar are some of the products that may be used as malt adjuncts.

A mixture of 20 to 35 per cent of malt adjuncts and 65 to 80 per cent of malt is commonly used in the manufacture of beers in this country.[1]

The following table will illustrate the kinds and quantities of raw materials used in the production of fermented malt liquors:

TABLE 20.—KINDS AND QUANTITIES OF RAW MATERIALS USED IN THE PRODUCTION OF FERMENTED MALT LIQUORS[1]

State	Malt, pounds	Corn and corn products, pounds	Rice, pounds	Sugar and sirups, pounds	Hops and hop extract, pounds
New York	365,982,112	66,220,393	42,148,497	27,209,257	6,264,718
Pennsylvania	235,699,855	58,789,660	10,781,961	25,751,413	4,261,147
Wisconsin	239,514,307	70,466,649	7,043,580	11,077,989	3,407,251
Illinois	136,212,599	32,629,753	20,376,841	11,526,705	2,315,744
Ohio	156,037,399	39,044,584	11,922,048	9,140,955	2,624,301
Missouri	130,426,803	5,177,625	36,902,989	3,642,173	1,936,504
Michigan	122,296,664	29,899,930	18,059,725	3,251,405	1,985,824
Total, United States	2,176,928,721	423,066,228	230,745,621	188,028,513	37,004,749

[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue,* June 30, 1937.

Calculated on the basis of the figures given in the foregoing tables, the average quantities of the raw materials used in the production of each barrel of fermented malt liquors are as follows:

Material	Pounds
Malt	37.05
Corn and corn products	7.20
Rice	3.92
Sugar and sirups	3.22
Hops and hop extract	0.63

It will be observed that slightly more than 52 lb. of material are used in the preparation of each 31-gal. barrel. Of the total raw material going

[1] POZEN, M. A., Enzymes in Brewing, *Ind. Eng. Chem.*, **26**: 1127 (1934).

reduced. It is believed that amylophosphatase may also attack linkages other than phosphate linkages. The ability to produce liquefaction is, however, one of the principal functions of amylophosphatase.

Alpha-amylase and beta-amylase, diastatic enzymes dextrinize and saccharify starch after it has been liquefied. α-Amylase acts principally to dextrinize starch, while β-amylase saccharifies starch or dextrins.

According to Hanes,[1] α-amylase ruptures the 30-glucose starch macromolecule to yield dextrins, there being 3 to 17 glucose molecules in a dextrin. β-Amylase may attack the freed dextrins or the nonaldehydic end of the starch macromolecule and split off molecules of maltose. About 60 per cent of the starch is thus converted by β-amylase to maltose. The remaining unconverted portion is known as α-amylodextrin or erythrogranulose.

During germination, a proteinase brings about a breakdown in the proteins before diastatic activity is developed to any appreciable extent. Protein molecules are degraded to soluble products, among them peptides. These are further attacked by peptidases, of which there are at least two in germinating grain, and broken down to amino acids.

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into each barrel of beverage, malt constitutes approximately 71.2 per cent; corn and corn products, 13.85 per cent; rice, 7.55 per cent; sugar and sirups, 6.19 per cent; or, in other words, the malt adjuncts represent 27.59 per cent of the total. In addition, hops and hop extract make up 1.21 per cent. These are, of course, average figures, based on nation-wide production and are only intended to convey a broad picture of the malt-beverage industry.

Mashing.—The purpose of mashing is to digest and to dissolve as much as possible of the valuable portions of the malt or malt adjuncts.

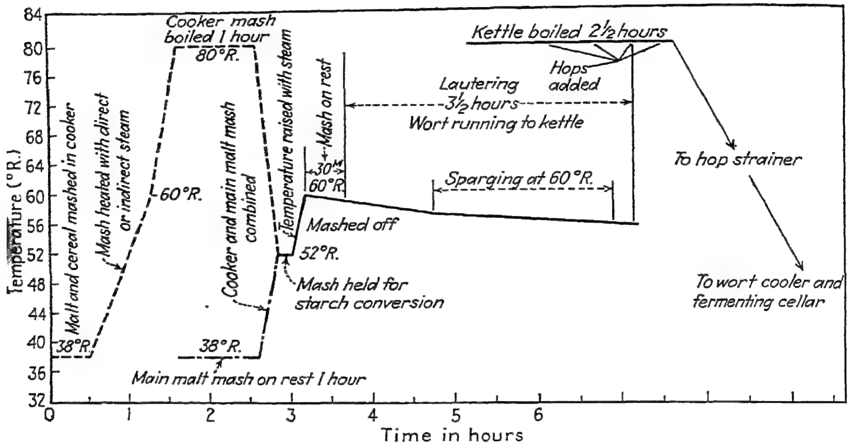

FIG. 14.—Time-temperature chart of brewhouse operations. (*Courtesy of Dr. R. Schwarz, Ind. Eng. Chem.*, **27**: 1031 (1935).)

The sweet wort that results from this enzyme process contains dextrins, maltose, other sugars, pentosans, protein degradation products, minerals, tannin, coloring matter, and other substances.

Mashing Methods.—There are two main methods for mashing: the infusion and decoction methods. Many modifications of these methods are used in practice.

THE INFUSION METHOD.—There are two infusion processes: one of these is the upward method; the other, the downward method. In the upward process, the malt is mixed (doughed in) with water at a temperature of 38 to 50°C. This main mash is permitted to rest (protein rest period) for about 1 hr. at this temperature to favor the action of the proteolytic enzymes. The temperature is then raised to 65 to 70°C. by the doughing in of the cooked starchy malt adjuncts, which are at the boiling temperature. The mash is permitted to stand at this temperature for a few minutes for starch saccharification. The temperature is then

brought to about 75°C., or a little above, for the destruction of the enzymes. The mash is filtered at this temperature.

In the downward infusion process, the initial temperature of the mash water may be about 77°C. The added malt aids in cooling the water to approximately 70°C. A temperature of 65 to 70°C. is maintained, as in the upward infusion process, to permit saccharification. The final temperature of the mash is lower than the initial temperature. The downward infusion process is an English method.

THE DECOCTION METHOD.—In this method, the mash is mixed at a lower temperature, occasionally around 40°C., than is the case with infusion processes. The temperature of the mash is raised by steps until a final temperature of about 75°C. has been obtained.

A portion of the initial mash, approximately one-third, is withdrawn, heated, and boiled for a short period of time and then returned to the main mash. The heated portion raises the temperature of this entire mash. The enzymes in the boiled portion have been destroyed, but the cell walls of the grain are softened and the starch liquefied. Diastatic action is thus facilitated by this process.

The mash may be maintained for a while at the new temperature. Thereafter another portion of the mash may be removed, boiled, and returned to the main mash. The process may be called a one-, two-, three-mash process or single-, double-, or triple-mashing process, depending on the number of portions that have been heated and returned to the mash.

In a three-mash process, according to Hopkins and Krause, the mash may be held at the initial temperature (40°C.) in order to extract the enzymes and favor proteolysis and the action of phytase. The mash may then be held at 50°C. for "full proteolysis and peptonization"; at 60 to 65°C. for saccharification; and at 70 to 75°C. for dextrinization of any unconverted starch by α-amylase and complete extraction.

Comparison of Methods.—The decoction process usually yields a higher percentage of extract than the infusion process. But the flavor of the wort obtained from the infusion process may be better than that of the decoction process, because bitter resins are not dissolved to such an extent. Either method should produce satisfactory results, however.

Important Considerations in Mashing.—In mashing, the proper preparation of the malt and uncooked malt adjuncts; the treatment of the mash water, if necessary; and the control of temperature, pH, mash concentration, and time are important.

Preparation of Malt.—Malt is selected, cleaned, and ground. Grinding, to be satisfactory, must be accomplished according to the variety of malt and the type of equipment being used. There should be proper distribution of the ground malt into husks, coarse grits, fine grits and

flour. The presence of unground kernels results in a loss of extract, while the production of too finely pulverized malt interferes with the filtration of the wort. The properly prepared malt is mixed with a measured quantity of water at the correct temperature in the mash kettle.

Treatment of Malt Adjuncts.—Unmalted cereals, such as corn meal, corn grits, rice, or wheat are mixed with water and a small amount of malt, agitated and brought gradually to a boiling temperature. The mash, known as a converter mash, is then cooked with or without the use of pressure to gelatinize the starch and render it soluble. The converter mash is eventually added to the main mash containing the malt.

Malt adjuncts that exist in the prepared state, such as cereal flakes, are added directly to the main mash without any preliminary cooking. Sugars and sirups are usually added to the wort.

Composition of Mash Water.—The composition of the water that is to be used in brewing is very important. The quantity and character of the dissolved salts are of much significance while pH is of secondary interest. Water containing calcium and magnesium sulphates with a permanent hardness of between 200 to 300 parts per million (expressed as calcium carbonate)[1] is usually suitable. On the other hand, soft water is required for the brewing of certain light lager beers. Filtration, clarification, and stability are influenced to some extent by the type of water used. "Burtonizing" is the name given to a process of artificially increasing the permanent hardness of water for ale and beer-making. It is so called from Burton-on-Trent in England where excellent ales have long been produced and the water supply is regarded as practically perfect for this purpose. (For a further discussion of this subject, the reader is referred to "Biochemistry Applied to Malting and Brewing," by Hopkins and Krause.)

Temperature.—The temperature of mashing is a very important factor in determining the type of beer to be produced. A high temperature is more favorable to the action of α-amylase than of β-amylase. Hence a higher proportion of dextrin will be produced when the mash is converted at a relatively high temperature. The fermentation of a wort containing a relatively large proportion of dextrin results in a low-alcohol beer. On the other hand, the use of a low conversion temperature favors the production of a larger proportion of sugar and less dextrin and, subsequently, a high-alcohol beer, or ale.

When the action of α-amylase is favored and that of β-amylase is inhibited, dextrins will accumulate at the expense of maltose. Dextrins are colloidal and, when present in a wort in sufficiently high concentration, will cause a slow filtration of the wort. Sparging will likewise be difficult, and considerable extract will be left in the spent grains. Furthermore,

[1] Schwarz, R., Brewing Processes, *Ind. Eng. Chem.*, **27**: 1031 (1935).

dextrins are not fermented by beer yeasts. It is this fact that permits one to control the alcohol in a given beer more readily.

The following table illustrates the effect of the temperature of conversion on the ratio of sugars to dextrins:

TABLE 21.—EFFECT OF THE TEMPERATURE OF CONVERSION ON THE RATIO OF SUGARS TO DEXTRINS[1]

Conversion Temperature, Degrees Centigrade	Ratio of Sugar to Nonsugar
64	1:0.37
66	1:0.40
68	1:0.48
70	1:0.52
72	1:0.57

, M. A., Enzymes in Brewing, *Ind. Eng. Chem.*, **26**: 1127 (1934).

By carefully controlling the temperature, it is thus possible to regulate the relative proportions of sugar to dextrins.

Starch liquefaction is most rapid at 70 to 75°C., while saccharification proceeds most rapidly at approximately 65°C. The optimum temperature for a given conversion is governed by the pH of the mash, its concentration, and other factors.

Temperature very definitely affects the type of proteolysis that occurs during mashing. At a conversion temperature of about 60°C., nitrogenous compounds of high molecular weight are formed. These compounds are valuable in the production of palatefulness, foam, and foam stabilization in beer. A greater proportion of amino nitrogen is formed at 50°C. The derivatives of low molecular weight, the amino acids in particular, are useful as food for the yeast in subsequent fermentation. An undue amount of proteolysis, especially the production of amino nitrogen in quantities greatly in excess of those required for the nutrition of the yeast, should be avoided.

pH.—By properly adjusting the pH of the mash, the quantity of extract will be increased, while subsequent clarification and filtration will be facilitated. The pH of the mash will influence the activity of the enzymes, each of which functions best at a given pH, which is dependent also on the temperature, concentration and nature of the mash, and other factors.

According to Hopkins and Krause, the yield of extract in a mash is maximum at a pH of around 5 to 5.2. β-Amylase is very active at this pH as well as protease. Maltose formation and attenuation are best at a pH of approximately 5.5. This value is excellent for filtration also. At pH values below 5, amino nitrogen is formed at a maximum rate.

Tannin and bitter resins are dissolved from the husk more readily at higher pH values, since they are weakly acidic in nature. Color, likewise, is extracted better at higher pH values.

During mashing, the pH varies, but it may be in the neighborhood of 5.8 at the beginning of the process. Lager beer, when ready for cooling, may indicate a pH of about 5.2 to 5.5.

High-grade lactic, sulphuric, phosphoric, or other acids may be used to adjust the reaction of the medium. It is essential, however, to use an acid that will have no deleterious effect on the flavor of the beer or its value to the consumer.

Concentration of Mash.—Since mashing is an enzymic process, the concentration of the mash influences the quality and quantity of the extract.

Time.—In order to control the composition of the final mash, it is important to regulate the time permitted for mashing. The time allowed will be intimately connected with the other factors of enzyme reaction—temperature, pH, concentration of substrate and menstruum.

During the last part of mashing especially the grains and coagulated proteins will settle to the bottom of the mashing tub. Although some manufacturers permit settling to take place in this tub and filter the wort by allowing it to flow down through the layer of grains and coagulated proteins, the wort passing out through a false bottom, others convey the material from the mash tub to a lauter tub. The lauter tub is a special filtering tank with a false bottom. Those which are wide and shallow give the quickest and best results. The filtered wort should be clear.

The grains that remain after the wort has been drawn off are "spent." They are extracted or leached several times with water at a temperature of 75 to 80°C., preferably 75°C., the process being known as "sparging." The sparged grain should finally contain less than 1 per cent of residual soluble extract. It is used as cattle food, either in wet form or after drying.

The wort and spargings are placed in a brew kettle, usually made of copper, and the hops are added in the proportion of 0.55 to 0.9 lb. per standard beer barrel of 31 gal.

Boiling the Mash.—Wort containing hops is boiled for several reasons: to concentrate it, to sterilize it, to inactivate the enzymes, to extract soluble substances from the hops, to precipitate coagulated proteins and other substances, and to slightly caramelize the sugar. The addition of the spargings to the main wort dilutes it, making concentration desirable. The danger of the growth of undesirable microorganisms in the wort is lessened by sterilizing it and handling it under aseptic conditions. Inactivation of the enzymes aids in maintaining a wort of fairly constant composition for fermentation.

The substances extracted from hops include bitter acids and resins, essential oil, and tannin. The bitter acids and resins contribute to the palatefulness, colloidal stability, and head retention of the beer. The

bitter acids are humulon or α-bitter acid ($C_{21}H_{30}O_5$), and lupulon or β-acid ($C_{26}H_{38}O_4$), respectively. By oxidation and polymerization the acids may be converted to soft resins. Both the acids and their corresponding resins possess antiseptic properties and impart characteristic flavors to beer. Humulon, however, possesses the strongest bitter flavor and the greatest antiseptic action. A third resin, of little value to the brewing industry, is the hard gamma resin.

A large proportion of the antiseptic action of hops is lost during the drying of the hops, their storage before use, boiling them with the wort, cooling, fermentation, and storage of the beer in barrels.[1]

Some flavor is imparted to beer from the essential oil of hops. Since the essential oil is volatile in steam, most of it is lost during the boiling of the wort, unless the hops are added toward the end of the boiling process.

Tannins are extracted principally from the hops during boiling, but some are extracted from the malt. The tannins from barley possess an unpleasant taste. Hence their removal by reaction with the proteins of the wort, before the hops are added, is advantageous.

Tannin aids in the precipitation of some of the nitrogenous substances of the wort. Some of the tannin complexes precipitate out during boiling, but others[2] tend to become insoluble in the cold, giving rise to a chill haze, unless removed during subsequent cooling.

Tannins are negatively charged. They react readily with positively charged proteins, forming complexes that become less soluble as the temperature decreases. Tannins react less readily with electrically neutral proteins, *i.e.*, proteins that are unstable.

Boiling converts some of the tannin of the hops into phlobaphene. Protein phlobaphene forms also during the boiling of the wort, and, being insoluble in the hot wort, it precipitates out. Oxygen accelerates protein-phlobaphene formation.[3]

Agitation and circulation of the wort during boiling increases the amount of precipitation and hence the quantity of sludge formed. During cooling, agitation is also of much advantage in increasing the amount of precipitation. The greater the quantity of unstable compounds removed during boiling and cooling, the less likelihood there is of the formation of precipitates in the finished product.

After the wort has been boiled, it is filtered through a hop strainer to remove the hops and precipitated proteins. The wort may, or may not,

[1] WALKER, T. K., A Review of Ten Years' Research on the Antiseptic Constituents of Hops, *Jour. Inst. Brewing*, **38**: 198 (1932).

[2] LUERS, H., and C. ENDERS, Acidity and Protein Turbidities in Beer, *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **22**: 329 (1938).

[3] HOPKINS, R. H., Prevention Better Than Cure for Brewing Troubles, *Food Industries*, **10**: 74 (1938).

be passed into a tank located above the cooler where settling may be permitted for 0.5 to 1 hr. Some cooling takes place in this tank. The wort is then cooled by passing it over or through coolers, preferably with considerable agitation.

During cooling, the wort becomes aerated and the rH* may increase from 12 or 13 to 16 or 17.[1] Protein-tannin compounds precipitate out owing to their insolubility at the lower temperatures. A secondary precipitation is induced wherein proteins and hop resins are adsorbed on the surfaces of the protein-tannin compounds.

The rate at which the wort is cooled between the temperatures of 60 and 21.1°C. has a direct bearing on protein-tannin precipitation,[2] brighter worts being secured through quicker cooling rates.

Preventing contamination of the wort during cooling is essential.

Fermentation. *Yeasts.*—Strains of *Saccharomyces cerevisiae* are commonly used in the manufacture of beer. The selection of an appropriate strain is a most important factor in determining the character of the beer.

Brewery yeasts may be classified as top-fermenting yeasts, which are used in the manufacture of ale; and bottom-fermenting yeasts, which are used in the production of lager and other beers.

Saccharomyces carlsbergensis Hansen[3] is a bottom yeast, which has been used in the breweries of Copenhagen and elsewhere. The Saaz yeast and *S. monacensis* are typical bottom yeasts also.

Pitching the Wort.—The addition of yeast to a wort is known as "pitching." Only pure cultures should be used for such purposes.

Wort is placed in tanks located in cellars (not necessarily below the surface of the ground), and maintained at a temperature that depends on the type of beer being manufactured. Some worts are stored at 0 to 6°C.; others may be stored at slightly higher temperatures. The wort is pitched and fermentation follows.

Fermentation.—It is the practice in some breweries to pump the fermenting wort to another fermentation tank after the appearance of a ring of white foam (krausen stage) on the surface of the wort. In so doing, precipitated proteins, hop resins, and some yeast cells are left behind. Since carbon dioxide is used to carbonate beers, the use of

*This is a term which represents the "negative logarithm of the hypothetical hydrogen pressure in equilibrium with the oxidation-reduction system" being studied. M. Stephenson, "Bacterial Metabolism," 2nd ed., Longmans, Green & Company, New York, 1939.

[1] Hopkins and Krause, "Biochemistry Applied to Malting and Brewing," D. Van Nostrand Company, Inc., New York, 1937.

[2] Sippel, G. B., Recent Advances in Brewing Technology, *Food Research*, **3**: 269 (1938).

[3] Guilliermond, A., "The Yeasts" (translated and revised by F. W. Tanner), John Wiley & Sons, Inc., New York. 1920.

closed fermenters with arrangements for carbon dioxide collection is now practiced.

During fermentation the temperature rises several degrees. Modern fermenters are provided with cooling equipment to prevent the temperature from rising too high.

As the fermentation proceeds, various substances accumulate on the surface of the medium, including hop resins, yeast cells, and proteinaceous material. This scum may be removed after the fermentation starts to recede, in order to improve the quality of the beer.

Certain very definite changes take place in the wort during fermentation. At a particular point near the end of the fermentation the yeast flocculates and commences to settle. This change is known as a "break" and depends in part upon the pH for initiation. (Not all yeasts break, however.) A large proportion of the fermentable sugars are transformed to ethyl alcohol, carbon dioxide, glycerol, and acetic acid. Higher alcohols and acids are produced from protein and fat derivatives. Esters are formed from organic acids and alcohols. The pH of the mash drops gradually. Insoluble products precipitate out.

The fermentation of a bottom-fermenting yeast, which is usually carried out at 6 to 12°C., is ordinarily completed in 8 to 10 days, while that of a top-fermenting yeast is completed in a shorter time, 5 to 7 days, at a somewhat higher temperature, 14 to 23°C.[1] Beer at the end of this stage is known as "green" or "young" beer.

The beer resultant from the fermentation still contains undesirable substances in suspension. The elimination of the suspended materials by clarification and the use of low temperatures, and the production of "bouquet" in the beer are brought about by subsequent processes.

Maturing.—Beer is stored in refrigerated cellars for periods of 2 weeks to several months, depending on the process being used and the type of beer desired. The temperature is maintained at 0°C. During storage, unstable proteins, yeast, resin, and other undesirable substances precipitate from the beer. The harshness of the "green" beer disappears. Esters are produced, and the beer becomes mellow or matured.

Finishing.—The matured beer is carbonated under pressure, using carbon dioxide that is at least 99.5 per cent pure, until a final carbon dioxide content of 0.45 to 0.52 per cent is obtained. Gas is absorbed by the beer, and oxygen is displaced. Exclusion of air in the finished beer is very important. The greater the amount of air displaced from the beer, the better. The dissolved gas adds to the quality of the beer, aids in the production and retention of foam, and helps to preserve the beer.

An alternate process for carbonating beer is known as the "krausening process." The beer after storage is placed in pressure tanks or con-

[1] *loc. cit.*

tainers, and approximately 15 per cent of beer in an initial active stage of fermentation (krausen) is added and mixed with it. The carbon dioxide evolved from the fermenting young beer impregnates the entire tank of beer. Valves or bungs, attached to the pressure tanks, enable one to regulate the final content of carbon dioxide in the beer (0.45 to 0.52 per cent). The krausening process usually requires 3 or 4 weeks. Afterward the beer is stored for an additional period of 3 to 8 weeks.[1] The action of the yeast stops after the sugars have been consumed. Clarification then proceeds.

Carbonated beer is cooled, filtered, and racked. By racking is meant the distribution of the filtered beer into bottles, cans, or other containers. During the first few months of the year 1937, approximately 43 per cent of the beer was packaged. Bottled beer is usually pasteurized for 20 min. at a temperature of 60 to 61°C.

Defects in Beer.—*Turbidities* in beer may be caused by a number of factors: unstable protein,[2] protein-tannin complexes, starch, resin, and microorganisms.

Gluten, or albumin, turbidity occurs at a low temperature generally. This type of turbidity is likely to appear when the malt has been improperly dried in the kiln or when a barley with very high protein content is used. Warming the beer causes the turbidity to disappear.

Oxidation turbidity or haze is due in part to protein-tannin compounds. The presence of oxygen; the shaking of the beer during its transportation; the collisions of beer bottles, which impart supersonic and high-pitched sound vibrations; and sunlight affect the formation of oxidation haze. Saturation of the beer with carbon dioxide does much to prevent this turbidity.

Tannin-protein hazes appear also at low temperatures. In order to produce stable, chillproof beers, which will not become hazy or turbid when cold, the use of a small amount of a proteolytic enzyme preparation is advantageous. The enzymes are usually added after the fermentation although they may be added earlier. The enzymes in an acid medium, such as is found in a beer, render the beer stable and chillproof. Credit is due to Wallerstein[3] for this discovery.

Starch turbidities develop as a result of the improper conversion of starch during mashing. Lack of proper digestion at this time may be due to the use of a malt in which the diastase has been destroyed during the kilning. Sparging with water at a temperature much higher than 80°C. may also result in the production of turbidity. Amylases may be added to the storage vats to remove starch turbidity.

[1] Schwarz, *loc. cit.*

[2] Luers and Enders, *loc. cit.*

[3] Wallerstein, L., U.S. Patents 995,820 and 995,824, 1911.

The presence of resin oil containing pitch may, rarely, cause turbidity, as may calcium oxalate. Proper filtration will prevent both types of turbidities.

Yeast turbidity may be due to lack of proper clarification during the secondary fermentation, which in turn is caused by an unsatisfactory wort. The use of chips, or krausening (the addition of beer in an active state of fermentation) is usually effective in correcting this type of turbidity. Wild yeasts, especially of the *Saccharomyces pastorianus* III species, produce turbidity. By excluding air and keeping the concentration of fermentable sugars in the beer low, growth of yeasts is inhibited. A low rH, 12 or below, will also inhibit the growth of yeasts according to De Clerck.[1] The use of pure cultures and proper sanitation of the plant should prevent the access and development of wild yeasts.

Among the turbidities caused by bacteria, those produced by sarcinae are most common, especially in bottom fermentations. Bacterial turbidities are less common than turbidities caused by yeasts and frequently will contain yeasts as well as bacteria. Aseptic technique, the use of pure cultures of yeasts, sanitation of the brewery, and a high content of hop antiseptics should prevent bacterial turbidities.

Faulty beer may be the result of the use of a low-grade raw material in the mash; the use of hops of poor quality, too much hops, or prolonged boiling of the hops; contact of the beer with iron, causing an inky taste; contact with tin; an unsuitable brewing water; young or green beer; or carbon dioxide deficiency; etc.

Definitions.

Lager beer is literally stored beer. The term "lager" is derived from "lagern," a German verb meaning "to store." According to this definition, all beer would be lager beer. Lager beer is produced by bottom fermentation and is rather high in alcohol and extract with a relatively low proportion of hops.

Bock beer is a heavy beer, dark in color and high in alcohol. It is brewed for consumption in the early spring.

Ale is produced by top fermentation, is pale in color, tart in taste, high in alcohol and contains more hops than beer.

Porter is a dark ale, high in extract and sweeter than the usual ale in taste. It is brewed from a dark or black malt (malt roasted at a high temperature) to produce a wort of high extract. The flavor of hops is less distinct than that of normal ale.

Stout is a strong porter that is high in alcohol and extract. It is dark in color and possesses a sweet taste and strong flavor of malt. The hop flavor is more pronounced than that of porter.

[1] De Clerck, *Jour. Inst. Brewing*, **40**: 407 (1934).

Weiss bier, a beer made mainly from wheat, is produced by top fermentation. It is rather light, possesses a marked flavor of malt and hop, is tart, and contains a large quantity of natural fermentation gas. It is likely to be turbid in appearance.

Cereal beverage is beer containing less than 0.5 per cent alcohol. It is sometimes known as "near beer."

Brewery Thermometers.—The brewer frequently uses the Reaumur thermometer instead of the Fahrenheit or centigrade scales. A comparison of a few points on the three scales is given in the following table:

TABLE 22.—A COMPARISON OF SOME POINTS ON THE REAUMUR, CENTIGRADE, AND FAHRENHEIT SCALES

Reaumur	Centigrade	Fahrenheit
−32	−40	−40
−16	−20	− 4
0	0	32
4	5	41
8	10	50
16	20	68
32	40	104
48	60	140
52	65	149
56	70	158
80	100	212

It will be noticed that the Reaumur degree is equivalent to 1.25 centigrade degrees.

Regulations.—Regulations concerning malt beverages are published by the Federal Alcohol Administration of the U. S. Treasury Department. The labeling and advertising of malt beverages, for example, are considered in Regulations 7 of the department.

Additional Information.—Further information concerning brewing may be obtained by referring to the publications listed at the end of the chapter. The texts of Hopkins and Krause and of Hind will be found to be of special value.

Some Other Alcoholic Beverages.—Alcoholic beverages are consumed in every country of the world. In some countries the use of a particular beverage has been passed down from antiquity, for example, kvass in Russia, pulque in Mexico, taette in Scandinavia, and sorgho in Manchuria. A brief description of a few alcoholic beverages follows:

Kvass may be prepared by mixing equal parts of barley malt, rye malt, and rye flour, adding boiling water, stirring, permitting the mash to

stand for several hours, adding more boiling water, and then inoculating with yeast and permitting fermentation to take place. Peppermint is added to the fermented product for flavoring. This beverage is very common in Russia. Neighboring countries prepare similar drinks in which other starchy or saccharine materials may be used.

Pulque is prepared by allowing the sweet juice of agave (the century plant is a common species) to undergo a spontaneous fermentation, which is usually complete in about 1 day. Yeasts produce alcohol from the sugars. Bacteria are present, which cause a rapid spoilage of the beverage unless it is consumed shortly after its manufacture. Pulque resembles cider and has a flavor somewhat similar to that of sour milk. It is a very common beverage in Mexico.

Taette is an alcoholic beverage prepared from milk. Yeasts cause the characteristic changes in flavor. The product has a pleasing acid taste.

Sorgho is an alcoholic drink made from *Sorghum saccharatum*.

Sake is the widely used alcoholic beverage of the Japanese. It is a yellow rice wine containing 14 to 24 per cent of alcohol. There are various methods by which it is manufactured. Usually a *koji*, known as *sake-koji* or *tane-koji*, is prepared. Steamed rice is inoculated with the spores of *Aspergillus oryzae* and incubated at about 20°C. until the rice is well covered with mycelium. The *koji*, or starter, is mixed with steamed rice and some water and inoculated at a low temperature. Starch is converted to fermentable sugars. The thick liquid resultant from this enzyme hydrolysis and in which spontaneous fermentation usually takes place is known as *moto*. *Koji*, *moto*, and more water are usually mixed. An alcoholic fermentation ensues in which several yeasts may be active. *Saccharomyces sake*, *S. tokyo*, and *S. yeddo* are some of the yeasts characteristic of sake.

Pombe is an alcoholic beverage made by permitting millet seed to sprout and undergo a conversion of the starch to sugars and by allowing a spontaneous fermentation of the saccharified starch in water.

Biti is a wine made from the tubercles of *Osbeckia grandiflora*. It is a West African drink.

Ginger beer is characterized by its distinctly acid nature, the ginger flavor, and the presence of a small amount of alcohol. Carbon dioxide is evolved in considerable quantity. The raw materials are sugar (in 10 to 20 per cent concentrations) and pieces of ginger root. "Ginger-beer plant" is added to a solution of the sugar. In the ginger-beer plant are a yeast, *Saccharomyces pyriformis*, and a bacterium, *Bacterium vermiforme*. The yeast cells are entangled in the gelatinous sheaths of the bacteria. A symbiotic relationship apparently exists, for both the yeast and bacterium function best when in each other's presence.

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Brewers Journal (formerly *Western Brewer*) (monthly), H. S. Rich & Co., Chicago, 1876–

Brewery Age (monthly) Brewery Age Publishing Co., Chicago, 1932–

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CHAPTER VI

WINE

Wine is the product made by the "normal alcoholic fermentation of the juice of sound, ripe grapes, and the usual cellar treatment."[1]

Classifications and Definitions.—Wines may be classified in many ways.

Dry wine is "wine in which the fermentation of the sugars is practically complete."[1] Most dry wines contain a small amount of sugar even though the quantity may be so slight as to escape detection by the sense of taste.

Sweet wine is "wine in which the alcoholic fermentation has been arrested."[1] Such wines contain sufficient sugar for taste perception. Wines may be fortified by the addition of brandy or wine spirits.

Fortified dry wine is "dry wine to which brandy has been added but which conforms in all other particulars to the standard of dry wine."[1]

Fortified sweet wine is "sweet wine to which wine spirits have been added."[1]

Sparkling wine is "wine in which the after part of the fermentation is completed in the bottle, the sediment being disgorged and its place supplied by wine or sugar liquor and/or dextrose liquor, and which contains, in 100 cc. (20°C.), not less than 0.12 g. of grape ash."[1] Such wine contains considerable carbon dioxide.

Wines may be red or white wines. A red wine is one containing the red coloring matter extracted from the skins of the grapes; a white wine is one "made from white grapes or the expressed fresh juice of other grapes."[2] In making a red wine, the skins and seeds are usually left with the must during fermentation.

"Modified wine, ameliorated wine, corrected wine is the product made by the alcoholic fermentation, with the usual cellar treatment of a mixture of the juice of sound, ripe grapes with sugar and/or dextrose, or a sirup containing not less than 65 per cent of the sugars, and in quantity not more than enough to raise the alcoholic strength after fermentation to 11 per cent by volume."[2]

Raisin wine is the "product made by the alcoholic fermentation of an infusion of dried or evaporated grapes, or of a mixture of such infusion or of raisins with grape juice."[2]

[1] U.S. Dept. of Agriculture, F. D. A., Service and Regulatory Announcements, Food and Drug, No. 2, Rev. 5, November, 1936.

[2] *Ibid.*

Regions of Production.—A large part of the world's wine is produced in the countries located near the Mediterranean Sea. France leads the world in the manufacture of wine, followed by Italy and Spain. Portugal, Greece, the Balkan States, and Germany; Algeria and other regions of North Africa; Chile and Argentina; Australia, Canada, and the United States produce considerable quantities of wine.

There are three principal regions of wine production in the United States, represented by (1) California; (2) Louisiana, Arkansas, and Missouri; and (3) New York, Ohio, New Jersey, and Michigan.[1]

Imports.—The imports of sparkling and still wines with their respective values for the years indicated are shown in Table 23.

TABLE 23.—AMOUNT AND VALUE OF IMPORTS OF SPARKLING AND STILL WINES[1]

	Quantity, thousands of gallons			Value, thousands of dollars		
	1934	1935	1936	1934	1935	1936
Sparkling wines	395	277	502	2,971	2,100	3,444
Still wines	3,463	2,494	3,134	10,212	6,681	8,021

[1] U.S. Dept. of Commerce, Statistical Abstract of the United States, 1937.

During the Government fiscal year 1935, the United States imported 803,000 gal. of wine from France, 120,000 gal. from Italy, 399,000 gal. from Spain and 133,000 gal. from Germany.[2]

Production Statistics.—Table 24 gives the production of still wine and the number of bonded wineries in the seven leading states and the United States during the fiscal year 1937.

A perusal of this partial table indicates that California produces most of the still wine manufactured in the United States. New York follows California as the second most important state.

Sparkling wine production by eight leading states during the fiscal year 1937 was as indicated in Table 25.

Chemical Composition of Wines.—In Table 26 are shown the chemical analyses of some wines of American origin made by wine makers receiving awards at the Paris Exposition during the year 1900. The data presented in the table were compiled and computed from the analyses given by Wiley. For further details, the reader is referred to the bulletin cited.

Volatile Acids.—Acetic and propionic acids are the volatile acids found in sound wines. Acetic acid is the principal volatile acid of young wines,

[1] GORESLINE, H. E., Notes on Wine Manufacture in the United States, *U.S. Dept. of Agr.*, mimeographed sheets, 1936.

[2] U.S. Dept. of Commerce, "Foreign Commerce Yearbook 1937," Washington, 1938.

but old wines contain traces of propionic acid in addition.[1] Formic acid is usually found in diseased wines, together with acetic acid.

Fixed Acids.—Tartaric, malic, and tannic acids are constituents of the must and are, therefore, present in the wine in varying proportions.

TABLE 24.—PRODUCTION OF STILL WINE AND NUMBER OF BONDED WINERIES IN LEADING STATES AND IN THE UNITED STATES, FISCAL YEAR 1937[1]

State	Bonded still wineries[2]	Production,[3] wine gallons
California	630	115,338,166
New York	123	3,147,822
Washington	35	956,860
Ohio	130	755,175
Michigan	11	479,609
Georgia	12	334,815
Louisiana	11	315,358
Total, United States	1,206	122,045,241

[1] *U.S. Treas. Dept., Annual Report of the Commissioner of Internal Revenue,* 1937.
[2] Number operated during any part of the year.
[3] Production represents the amount removed from fermenters, including wine that is removed for use as distilling material in the production of brandy.

Much of the tartaric acid is removed during the manufacture of wine, especially during the fining process.[2]

TABLE 25.—PRODUCTION OF SPARKLING WINE, BY LEADING STATES, FISCAL YEAR 1937

State	Production, Half-pint Units
New York	4,652,321
California	1,531,358
New Jersey	1,502,366
Missouri	984,022
Ohio	736,705
Michigan	171,745
Washington	41,312
Pennsylvania	2,696
Total, United States	9,622,525

Citric acid[3] is found in some grapes and in some wines. Succinic acid is produced in very small quantities during yeast fermentation. Phosphoric acid is a natural constituent of wine.

[1] MORRIS, M. M., Volatile Acids of Wine, *Ind. Eng. Chem.*, **27**, 1250 (1935).
[2] WOODMAN, A. G., "Food Analysis," 3d ed., McGraw-Hill Book Company, Inc., New York, 1931.
[3] WINTON, A. L., and K. B. WINTON, "The Structure and Composition of Foods," Vol. II, John Wiley & Sons, Inc., New York, 1935.

TABLE 26.—CHEMICAL ANALYSES OF AMERICAN WINES RECEIVING AWARDS AT PARIS IN 1900[1]
(Grams per 100 cubic centimeters)

	Sparkling wines			Dry white wines			Dry red wines			Sweet white wines			Sweet red wines		
	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average
Specific gravity at 15.5°C.	1.0169	0.9910	1.0045	0.9939	0.9901	0.9917	0.9969	0.9926	0.9943	1.0494	0.9908	1.0298	1.0522	1.0107	1.0276
Alcohol by volume, per cent.	15.20	11.65	13.22	14.25	10.60	12.45	15.40	10.10	12.61	21.55	11.60	18.38	22.05	13.70	19.30
Alcohol	12.06	9.25	10.48	11.31	8.41	9.88	12.22	8.01	10.00	17.10	9.21	14.58	17.50	10.87	15.31
Glycerol	0.7330	0.2301	0.4177	1.0119	0.5812	0.7019	0.9504	0.5341	0.6355	0.7350	0.0483	0.3025	0.7460	0.2936	0.5089
Glycerol-alcohol ratio	....	....	....	9.2:100	5.7:100	7.1:100	81:100	5.8:100	6.4:100						
Extract	8.56	1.78	5.40	2.51	1.55	1.99	3.22	1.77	2.57	19.45	2.83	13.80	19.71	7.57	13.52
Ash	0.290	0.114	0.153	0.270	0.107	0.196	0.393	0.138	0.247	0.263	0.097	0.203	0.374	0.234	0.311
Total acids	0.783	0.601	0.658	0.715	0.433	0.586	0.901	0.454	0.649	0.805	0.160	0.412	0.826	0.397	0.502
Fixed acids	0.715	0.418	0.566	0.570	0.326	0.459	0.690	0.346	0.507	0.528	0.116	0.300	0.508	0.257	0.472
Volatile acids	0.148	0.049	0.082	0.174	0.050	0.101	0.266	0.071	0.128	0.222	0.029	0.092	0.255	0.080	0.122
Total tartaric acid	0.357	0.163	0.271	0.352	0.119	0.189	0.252	0.083	0.163	0.296	0.038	0.142	0.145	0.025	0.078
Free tartaric acid	0.141	0.000	0.065	0.1785	0.0000	0.0677	0.0468	0.000	0.000	0.1436	0.000	0.0166	0.000	0.000	0.000
Polarization, °V	−13.3	−1.8	−5.2	−1.1	−0.1	−0.5	−0.8	0.0	−0.4	−25.7	0.0	−11.8	−19.4	−7.7	−16.4
Reducing sugars	5.584	0.023	3.409	0.328	0.051	0.134	0.280	0.045	0.146	16.910	1.767	11.30	16.960	3.240	10.26
Protein	0.698	0.070	0.214	0.337	0.060	0.162	0.305	0.077	0.150	0.245	0.026	0.162	0.368	0.105	0.232
Potassium sulphate	0.128	0.014	0.033	0.13	0.043	0.087	0.133	0.016	0.070	0.088	0.007	0.044	0.062	0.024	0.048
Phosphoric acid	0.026	0.012	0.017	0.066	0.010	0.022	0.044	0.015	0.029	0.059	0.008	0.034	0.068	0.018	0.045
Free sulphurous acid	0.0031	....	....	0.0078	0.0002	0.0017	....	....	....	0.0059					
Total sulphurous acid	0.0456	0.0010	0.0074	0.0163	0.0013	0.0063	....	....	....	0.0188	0.0011	0.0045	0.0047		
Tannin	0.066	0.009	0.035	0.0830	0.0178	0.0391	0.3435	0.1388	0.2364	0.0662	0.0213	0.0363	0.2207	0.0516	0.0962

[1] WILEY, H. W., American Wines at the Paris Exposition of 1900: Their Composition and Character, *U.S. Dept. Agr., Bur. Chem., Bull.* 72, 1903.

Wine Manufacture.—Although the fundamentals of wine making are similar in all three of the principal regions in the United States, methods vary to some extent in the different regions owing to the varieties of grapes used, the climate, and other factors. For example, a lower temperature may be used in the pasteurization of wines produced in the eastern portion of the United States on account of the greater acidity of the wine.

In view of the magnitude of the industry in California, special attention will be devoted to discussing wine making as it is practiced there, illustrating with a description of the production of red wine.

The reader desirous of securing additional knowledge of wine making will find numerous references at the end of this chapter, which should prove to be both interesting and instructive.

The Manufacture of Red Wine. *Outline of the Process.*—Selected grapes of the proper maturity are crushed and stemmed; treated with sulphur dioxide, or a sulphite, or pasteurized; and inoculated with a starter containing a pure culture of yeast. After a short fermentation period the wine is drawn off, placed in storage tanks for further fermentation, racked, stored for aging, clarified, and packaged.

Details of the Process. 1. THE GRAPE.—The production of a fine wine may be regarded as commencing with the selection of the best variety of grape for use in its manufacture. Bioletti[1] recommends the following varieties of grapes for the production of red wine: Beclan, Blue Portuguese, Cabernet Sauvignon, Carignane, Gros Verdot, Merlot, Mondeuse, Petite Sirah, Serine, Tannat, and Zinfandel for the coast counties of California; and Barbera, Grenache, Gros Mansenc, Lagrain, Pagadebito, Refosco, St. Macaire and Valdepēnas for the interior valleys of California.

The quality of the grapes of a given variety will depend upon the conditions under which they are grown—soil, climate, and other conditions.

Grapes should be gathered at the proper stage of maturity. In order to determine the degree of maturity, representative bunches of grapes are picked, and the Balling degree of their juice is determined. A reading of 21 to 23° Balling is usually given by the juice of the grapes when they are at the optimum stage of maturity.[2]

2. HANDLING THE GRAPES.—In gathering the grapes and transporting them to the winery, the prime purpose should be to have them arrive in the very best condition possible. The grapes should be picked with care, placed in clean containers, and protected from deterioration. Careful supervision of the handling of grapes is essential.

3. CRUSHING THE GRAPES.—Grapes are crushed and stemmed by machine. The chemical composition of the metal used in the construction

[1] BIOLETTI, F. T., *Calif. Agr. Ext. Circ.* 30, Revised, 1934.

[2] JOSLYN, M. A., and W. V. CRUESS, *Calif. Agr. Ext. Circ.* 88, November, 1934.

of this machinery and other equipment about the winery is important. Iron and steel are used in some wineries but are undesirable for they may cause clouding of the wine, forming so-called "ferric casse." The tin and copper dissolved from bronze by grape juice, if sufficient in quantity, may cause flavor and color losses during the aging process. Stainless steel, nickel or inconel should be used in preference to iron, ordinary steel, and many bronzes.

If the grapes are not picked when cool, it is desirable to permit them to cool overnight before they are crushed.

4. TREATMENT BEFORE FERMENTATION.—Grapes contain on their surfaces a varied flora of microorganisms—molds, yeasts, and bacteria. It is quite possible that the juice of crushed grapes will produce a good wine without any special precautions, but a wine manufacturer cannot afford to gamble in respect to the quality of his wine. He can do much to insure the quality of his final product by destroying or inhibiting the development of the microorganisms found on the grapes and by the use of starters containing pure cultures of the specific yeast desired.

Sulphur dioxide or sulphites destroy or inhibit the growth of many undesirable types of microorganisms—acetic acid bacteria, wild yeasts, and molds—with a minimum amount of injury to the true wine yeast. Usually 2 to 6 oz.,[1] or twice the quantity of potassium metabisulphite, are added per ton of crushed grapes, the quantity used depending on the condition of the grapes—their maturity, the degree of contamination with molds, the temperature of the crushed product, and other factors. The largest quantities are used when the grapes are overripe, moldy, or relatively warm.

Pasteurization may be used in place of sulphites but is not usually considered to be so desirable.

5. THE FERMENTATION.—The selection of a yeast, the nutrient substances in the must (grape juice), the concentration of the sugar, the acidity, the oxygen supply, and the temperature are factors that must be supervised in respect to fermentation.

Saccharomyces ellipsoideus is the yeast used for the fermentation of must. Selected strains, such as the Burgundy or Tokay strains, are frequently used. Many strains, bearing different names, are known.

A starter is prepared from a pure culture of the selected yeast. Pasteurized must is used as the culture medium in preparing the starter, the magnitude of which should represent 2 to 5 per cent of the volume of the crushed grapes being inoculated.

It is usually unnecessary to add any substances for the nutrition of the yeast since the crushed grapes are an adequate source of supply. On rare occasions ammonium sulphate or phosphates may be added.

[1] *Ibid.*

Joslyn and Cruess[1] state that the optimum concentration of sugar is 22° Balling. The use of much higher concentrations of sugar favors the production of more than 13 per cent of alcohol by volume. Since alcohol tends to inhibit the fermentation when present in concentrations of 13 to 15 per cent by volume, a maximum of 13 per cent is usually desirable. The concentration that actually inhibits fermentation depends in part on the temperature of fermentation, the tolerance of the yeast for alcohol decreasing with increasing temperature. The approximate concentration of the alcohol that will be produced in the wine can be predetermined by multiplying the Balling reading of the must by 0.575.

It is permissible to reduce the concentration of sugar in must by the addition of water.[2] Another practice is to mix the juice with the high sugar concentration with a juice of low sugar concentration. Occasionally sugar may be added to must.

Grapes that have been permitted to become too mature are frequently of low acidity. Fruit acid—tartaric, citric, or malic acids—may be added to restore the normal acidity.

A large supply of oxygen is essential for the rapid multiplication of yeast cells and the starting of the fermentation, as stated under yeast manufacture (Chap. VIII), while the later stage characterized by alcohol and carbon dioxide production rather than growth proceeds best under nearly anaerobic condition.

Approximately 6 hr. after treating the crushed grapes with sulphur dioxide or sulphite, the starter is added. Thereafter the contents of the tank are mixed, or stirred, at least twice a day, except during the main fermentation, to facilitate aeration, temperature equalization, and the extraction of color and tannin. Normally a "cap" forms on the surface of the fermentation vat, which contains grape skins, pieces of stem, seeds and other suspended matter. To mix the contents of the tank, one may punch down the cap or pump juice from the bottom of the vat over the surface of the must.

The amount of aeration produced by mixing the contents of the tank is determined by the effectiveness of the procedure and by the frequency at which the operation is repeated. Provided that the fermentation is slow at the beginning, or near the end of the incubation period, the supply of oxygen may be increased by more frequent mixing of the contents of the vat. However, the must should not be overaerated during fermentation, for overaeration is likely to produce a wine of inferior quality, especially insofar as color and flavor are concerned.

[1] *Ibid.*

[2] Cruess, W. V., "The Principles and Practice of Wine Making," The Avi Publishing Co., Inc., New York, 1934.

Fermentation should be carried out at carefully controlled temperatures. The finest wines are produced usually at temperatures below 85°F. (29.4°C.). The development of bouquet and aroma are favored by maintaining the fermenting must at low temperatures, around 70 to 75°F. (21.1 to 23.9°C.), for example. A temperature range of 70 to 90°F. (21.1 to 32.2°C.) is satisfactory. When the temperature rises to 85°F. (29.4°C.) or, at the most, to 90°F. (32.2°C.), the mash should be artificially cooled. Temperatures above 95°F. (35°C.) are considered unsafe, while the fermentation is inhibited usually at temperatures of 97 to 100°F. (36.1 to 37.8°C.). Fermentations cease at a temperature of 105°F. (40.5°C.)[1] generally. Undesirable bacteria develop at the higher temperatures. Accordingly the quality of the wine is impaired. Obviously, at too low temperatures, the fermentation is too slow to be practical.

During the fermentation, records of the temperature and the Balling degree should be made at least twice a day, one set of observations being recorded on the side of the fermentation vat in order that the progress of the wine may be followed.

After 3 to 5 days of active fermentation, sufficient tannin and a maximum of color have been extracted from the skin of the grape. Extraction is facilitated by the agitation of pomace (skin, seeds, and pieces of stems) during fermentation, by the ethyl alcohol produced from the grape sugar, the heat of fermentation, and the mechanical breaking up of the skin.

The wine maker decides when the color and tannin content are satisfactory and then draws off the wine to separate it from the pomace. He does not wait for all the sugar to be fermented. At the time of drawing off the wine, the Balling reading may be 0 to 4°. It is not considered advisable to mix the wine drawn off ("free-run wine") with that expressed from the pomace, for the latter is of lower quality.

6. FURTHER FERMENTATION.—The free-run wine is placed in closed storage tanks, equipped with bungs that allow the excess carbon dioxide to escape. An atmosphere of carbon dioxide over the wine tends to inhibit the development of acetic acid bacteria and other aerobic types of microorganisms. The fermentable sugar is usually consumed in 7 to 11 days at a temperature of 70 to 85°F. (21.1 to 29.4°C.).

If the after fermentation becomes sluggish before the sugar is utilized, the yeast may be activated by pumping over the wine.

7. RACKING.—By racking is meant the drawing off of the wine from the lees or sediment. Potassium bitartrate ($KHC_4H_4O_6$), *i.e.*, cream of tartar, is found in the lees. This substance is less soluble in alcohol than in water and precipitates out more rapidly at low temperatures.

Wine is racked to facilitate its clearing and to prevent undesirable flavors from being extracted from the old yeast.

[1] *Ibid.*

8. STORAGE AND AGING.—Two important changes take place during storage and aging: clearing of the wine and the development of flavor.

In a new wine there are present substances which, if not removed, will produce a sediment and probably cloudiness. These substances include tartrates, certain proteins, and other matter. Naturally these substances would be removed by racking and filtration during a somewhat long storage and aging process, but the modern trend is to hasten the removal of these substances by methods that involve flash pasteurization (to precipitate certain proteins), cooling to room temperature and then to 24 to 27°F. (−4.44 to −2.78°C.), and holding at the latter temperature for a few days. Filtration is carried out in the cold. Since the acid content of the wine is frequently reduced by the foregoing rapid process it is customary to adjust the acidity with citric or tartaric acids, the former acid being preferred. The wine is placed in tanks for aging.

Wine storage tanks are generally constructed of white oak or redwood, white oak being the better of the two. The tanks are completely filled with wine and sealed to prevent the access of large quantities of oxygen, which would favor the growth of acetic acid bacteria and *Mycoderma vini* (wine flowers). Some wine is always lost through evaporation. Consequently, the tanks should be inspected regularly and kept filled with wine. Periodically the wine is racked. During racking and filling, especially, carbon dioxide is lost while some oxygen is absorbed. A small amount of oxygen accumulates in the headspace over the wine.

Flavor, which is due to a combination of taste and odor, is developed in wine as a result of oxidative changes and ester formation.

Aging proceeds slowly until oxygen becomes available in small quantities. It is inhibited by the presence of large quantities of carbon dioxide, by sulphur dioxide, and by the exclusion of air. New wines stored in airtight bottles do not age properly.

Alcohols, aldehydes, tannins, and other substances present in the wine are oxidizable. Alcohols may be converted to aldehyde and subsequently to acids by oxidation. Aldehydes form acetals with alcohol.

Combinations of alcohols with acids give rise to esters, which are important in the production of aroma or bouquet. Although opinions differ concerning the importance of esters, it is recognized that the nature of volatile esters is of greater significance than the quantity.[1] The esters of acetic acid contribute much to the flavor and bouquet of wine.

The time required for aging varies with the type of wine and the conditions. A dry wine may require at least 2 years. Some fine wines are aged for 5 or more years.

9. CLARIFICATION.—Wines may clear naturally over a period of time, but resort is frequently made to the use of finings followed by filtra-

[1] NELSON, E. K., *Food Research*, **2**: 221 (1937).

tion, heating, refrigeration, or combinations of the foregoing. Fining agents, which include such substances as casein, gelatin and tannin, bentonite (of Wyoming origin, if possible), isinglass (fish protein from the sturgeon), white of egg, and Spanish clay, are mixed with the wine carefully according to direction, or preferably after laboratory tests have been carried out with small portions of the wine and the fining agents. The improper use of some of the fining agents may, in themselves, be a cause of clouding of the wine.

Filtration is carried out with filter aids. For further details the reader is referred to "The Principles and Practice of Wine Making" by Cruess and to the more recent publications on this subject.[1]

10. PACKAGING.—The clarified wine is placed in oak barrels for bulk sale and in bottles or in cans for unit sale.

Bottles of small and medium size may be pasteurized for 30 min. at 140°F.[2]

Defects of Wine.—Defects in wine may be caused by microorganisms, in which case they are known as diseases, or by other agencies. The diseases of wine are of two general types: those caused by aerobic microorganisms, and those caused by facultative anaerobes or anaerobes.

Diseases Caused by Aerobic Microorganisms.—The aerobic diseases of wines are caused principally by mycodermas and acetic acid bacteria. These microorganisms grow well in wine in the presence of oxygen. They cause no trouble if the wine is carefully supervised during its manufacture and storage. They are most likely to become active during the fermentation of must, if the cap is not punched down frequently, and during the storage of wine, if the containers are not kept properly filled and sealed.

Mycoderma vini (wine flowers) forms a film over the surface of wine and attacks the extract, the alcohol, and occasionally the organic acids.

Acetic acid bacteria will produce vinegar from wine in the presence of oxygen, unless they are destroyed or prevented from growing. Their activities are discussed in the chapter on vinegar.

Diseases Caused by Facultative Anaerobes or Anaerobes. TOURNE DISEASE.—The term "tourne" is considered to signify either the organism causing the disease or the condition produced in wine by large numbers of these bacteria.[3] Tourne is considered to be the most serious disease of wines and one of the most common.[4]

The organism is an anaerobic bacterium, which occurs as long, slender rods. It may be found in red or white wines, or in fortified wines with an alcohol content of 20 per cent or greater. It grows best, however, if the

[1] SAYWELL, L. G., *Ind. Eng. Chem.*, **27**: 1245 (1935).
[2] CRUESS, W. V., *Fruit Products Jour.*, **15**: 40 (1935).
[3] CRUESS, W. V., The Tourne Disease of Wine, *Fruit Products Jour.*, **14**: 198 (1935).
[4] JOSLYN and CRUESS, *op. cit.*

alcohol concentration is not too great. Sugar and other nutrient substances favor its growth. It is likely to develop in wines "stuck" due to high temperatures. It is inhibited slightly by tannin but very strongly by sulphur dioxide and metabisulphites.

Tourne is indicated[1] by increasing volatile acidity, by decreasing fixed acidity, by a "silky" type of cloudiness, and, when the condition has progressed well, by an odor and taste that is termed "mousey."

Tourne may be detected by a microscopic examination of the sediment, obtained by centrifuging a sample of the wine, or by analyses of the wine for volatile acids. If the maximum amount of volatile acid permitted in wine by law—0.14 for red wine or 0.12 for white wine—has been produced or exceeded, then there is good evidence that the wine may be infected with tourne. Taste may also be of some assistance in its detection.

The judicious application of sulphur dioxide, 75 p.p.m., to wines, or pasteurization; the use of a high degree of cleanliness about the plant; sterilization of equipment with steam when necessary; and rigid laboratory control should lower the incidence, or prevent, tourne disease of wine.

A pasteurization of bottled wine at a temperature of 145°F. for 30 min. is very effective in preventing tourne.

Once wine has been infected by tourne, it should be made brilliantly clear by filtration with selected infusorial earths, or by clarification with bentonite followed by passage through germproof filters.[2] Sulphur dioxide, or its equivalent of metabisulphite, should then be added to the wine in such quantity that its concentration will be maintained at 75 p.p.m., or greater. All equipment that has been infected should be treated with live steam or a suitable disinfectant to destroy the source of infection.

LACTOBACILLUS HILGARDII.—Spoilage of some dry wines in California has been caused by *Lactobacillus Hilgardii*,[3] a nonmotile, nonsporulating rod, which produces lactic and acetic acids. A silky cloudiness is produced, while the flavor is affected and becomes somewhat "mousey."

(MANNITE)-FORMING BACTERIA.—Bacteria that produce volatile acid, lactic acid, and mannitol ($C_6H_{14}O_6$) from glucose grow well at temperatures above 100°F. Their growth is inhibited by keeping the temperature of fermentation well below this point, by the use of sulphur dioxide, and by an increase in the acid content of the wine due to the addition of citric or tartaric acid.[1]

[1] JOSLYN and CRUESS, *op. cit.*

[2] CRUESS, W. V., *Fruit Products Jour.*, **14**: 198 (1935).

[3] DOUGLAS, H. C., and W. V. CRUESS, *Food Res.*, **10**: 113 (1936).

SLIME-FORMING BACTERIA.—Infected wine becomes slimy and cloudy. Slime formation occurs chiefly in white wines, usually young wines in closed containers. The occurrence of slime-forming bacteria in wine is not common and may be prevented by adding tannin to wines low in this substance and by the use of sulphur dioxide or metabisulphite.

OTHER BACTERIAL DISEASES.—Cocci, which may be inhibited by SO_2 or destroyed by pasteurization, may cause cloudiness in white wines.

Bitter wines may be caused by the growth of butyric acid bacteria, while a sour wine may result from the growth of lactic acid bacteria.

Defects Not Caused by Microorganisms.—Defects in wines may be caused by metals, enzymes, and the improper use of certain fining agents.

Iron is a cause of clouding in wines. Two different types of defects are produced by iron: one is known as black, blue, or ferric casse; the other as white casse.

Ferric casse is indicated by the appearance of a gray to gray-blue sediment in the wine and by clouding. The defect is found in bottled white wines, especially. Iron forms a precipitate with the tannin and coloring matter of red wine. Only a few parts of iron in a million parts of wine will produce ferric casse. Oxygen facilitates the formation of ferric ions from ferrous ions.

Ferric casse may be prevented by using equipment constructed of the proper types of metal and inhibited by 0.1 per cent citric acid.

The defect may be treated in one of several manners. In one method,[1] the iron is oxidized to the ferric stage by aeration. Tannin to the amount of 0.05 per cent is added. Clarification with casein and bentonite follow. In a second method, the iron is oxidized by aeration, tannin is added, and then gelatin. Settling is permitted, which is followed by racking, filtration, and acidification with citric acid. In a third method, tartaric acid is added to the wine, and the wine is then refrigerated. Cream of tartar and iron salts are precipitated.

White casse is also caused by an excess of iron. The precipitate is due in part to iron phosphate. It occurs in white wine. Treatment is as outlined above for ferric casse.

Oxidase casse is uncommon. It is caused by an enzyme, oxidase, produced by certain molds, which causes white wines to become brown and the color to be precipitated in red wines. Sulphur dioxide inhibits this oxidase, while pasteurization at 180°F. destroys it.

Other Defects.—Tin, tin salts, copper, aldehydes, or excessive quantities of gelatin may cause clouding of the wine. For a further discussion of this subject the reader is referred to the publications of Cruess and other workers.

[1] ... G., *Ind. Eng. Chem.*, **26**: 379 (1934).

Coatings of Wine Tanks.—Concrete tanks are used for the fermentation of must and the storage of wine, but they must be lined to prevent an undue amount of calcium from being dissolved by the wine. Steel tanks should be lined to prevent the solution of iron.

According to Cruess[1] and his associates one effective method for coating concrete is to treat the concrete first with a 0.5 per cent, then a 25 per cent, solution of tartaric acid. An insoluble calcium tartrate forms over the surface of the tank.

A mixture of 25 per cent Gilsonite and 75 per cent paraffin produces a protective coating for concrete and steel. Such linings are easily applied and not expensive.

Bass-Hueter black enamel gives good results[1] with both steel and concrete. A coating of paraffin over the enamel gives even better protection.

Standards of Identity for Wine.[2]—The Federal Alcohol Administration has set up standards of identity for wine, the text of which follows:

Article II. Sec. 20. *Application of standards.*—The standards of identity for the several classes and types of wine set forth herein shall be applicable to all regulations and permits issued under the act. Whenever any term for which a standard of identity has been established herein is used in any such regulation or permit, such term shall have the meaning assigned to it by such standard of identity.

Sec. 21. *The standards of identity.*—Standards of identity for the several classes and types of wine set forth herein shall be as follows:

Class 1. *Grape wine.*—(*a*) "Grape wine" is wine produced by the normal alcoholic fermentation of the juice of sound, ripe grapes (including restored or unrestored pure condensed grape must), with or without the addition, after fermentation, of pure condensed grape must, and with or without added fortifying grape spirits or alcohol, but without other addition or abstraction except as may occur in cellar treatment, *Provided,* That the product may be ameliorated before, during, or after fermentation by either of the following methods:

(1) By adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent; but in no event shall any product so ameliorated have an alcoholic content, derived by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

(2) By adding, separately or in combination, not more than 11 per cent by weight of dry sugar, or not more than 10 per cent by weight of water.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural red wine, more than 0.14 gram, and for other grape wine, more than 0.12 gram, per 100 cubic centimeters (20°C.).

[1] CRUESS, W. V., T. SCOTT, H. B. SMITH, and L. M. CASH, *Food Res.*, **2**: 385 (1937).

[2] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 4, Amendment 2, Washington, Aug. 22, 1938.

(*b*) "Red wine" is grape wine which contains the red coloring matter of the skins, juice, or pulp of grapes.

(*c*) "White wine" is grape wine which does not contain the red coloring matter of the skins, juice, or pulp of grapes.

(*d*) "Light wine" (including "light grape wine," "light red wine," and "light white wine") is grape wine having an alcoholic content not in excess of 14 per cent by volume.

(*e*) "Natural grape wine" (including "natural red wine" and "natural white wine") is grape wine containing no fortifying grape spirits or added alcohol.

(*f*) "Angelica," "madeira," "muscatel," and "port" are types of grape wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to these products, and an alcoholic content of not less than 18 per cent by volume.

(*g*) "Sherry" is a type of grape wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to this product, and an alcoholic content of not less than 17 per cent by volume.

(*h*) "Light port" and "light sherry" are types of grape wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to "port" and "sherry," respectively, and an alcoholic content of more than 14 per cent by volume.

Class 2. *Sparkling grape wine.*—(*a*) "Sparkling grape wine" (including "sparkling wine," "sparkling red wine," and "sparkling white wine") is grape wine made effervescent with carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle.

(*b*) "Champagne" is a type of sparkling light white wine which derives its effervescence solely from the secondary fermentation of the wine within glass containers of not greater than one gallon capacity, and which possesses the taste, aroma, and other characteristics attributed to champagne as made in the champagne district of France.

(*c*) A sparkling light white wine having the taste, aroma, and characteristics generally attributed to champagne but not otherwise conforming to the standard for "champagne" may, in addition to but not in lieu of the class designation "sparkling wine," be further designated as "champagne style" or "champagne type" or "American (or New York State, California, etc.) champagne—bulk process"; all the words in any such further designation shall be equally conspicuous and shall appear in direct conjunction with and in lettering approximately one-half the size of the words "sparkling wine."

Class 3. *Carbonated grape wine.*—(*a*) "Carbonated grape wine" (including "carbonated wine," "carbonated red wine," and "carbonated white wine") is grape wine made effervescent with carbon dioxide other than that resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle.

Class 4. *Citrus wine.*—(*a*) "Citrus wine" or "citrus fruit wine" is wine produced by the normal alcoholic fermentation of the juice of sound, ripe citrus fruit (including restored or unrestored pure condensed citrus must), with or without the addition, after fermentation, of pure condensed citrus must, and with or without added fortifying citrus spirits or alcohol, but without other addition or

abstraction except as may occur in cellar treatment, *Provided*, That the product may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent, but in no event shall any product so ameliorated have an alcoholic content, derived by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural citrus wine, more than 0.14 gram, and for other citrus wine, more than 0.12 gram, per 100 cubic centimeters (20°C.).

(*b*) "Light citrus wine" or "light citrus fruit wine" is citrus wine having an alcoholic content not in excess of 14 per cent by volume.

(*c*) "Natural citrus wine" or "natural citrus fruit wine" is citrus wine containing no fortifying citrus spirits or added alcohol.

(*d*) Citrus wine derived wholly (except for sugar, water, or added alcohol) from one kind of citrus fruit, shall be designated by the word "wine" qualified by the name of such citrus fruit, *e.g.*, "orange wine," "grapefruit wine." Citrus wine not derived wholly from one kind of citrus fruit shall be designated as "citrus wine" or "citrus fruit wine" qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Citrus wine rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle shall be further designated as "sparkling"; and citrus wine rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated."

Class 5. *Fruit wine*.—(*a*) "Fruit wine" is wine (other than grape wine or citrus wine) produced by the normal alcoholic fermentation of the juice of sound, ripe fruit (including restored or unrestored pure condensed fruit must), with or without the addition, after fermentation, of pure condensed fruit must, and with or without added fortifying fruit spirits or alcohol, but without other addition or abstraction except as may occur in cellar treatment, *Provided*, That the product may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent, but in no event shall any product so ameliorated have an alcoholic content, derived by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural fruit wine, more than 0.14 gram, and for other fruit wine, more than 0.12 gram, per 100 cubic centimeters (20°C.).

(*b*) "Berry wine" is fruit wine produced from berries.

(*c*) "Light fruit wine" is fruit wine having an alcoholic content not in excess of 14 per cent by volume.

(*d*) "Natural fruit wine" is fruit wine containing no fortifying fruit spirits or added alcohol.

(*e*) Fruit wine derived wholly (except for sugar, water, or added alcohol) from one kind of fruit shall be designated by the word "wine" qualified by the name of such fruit, *e.g.*, "peach wine," "blackberry wine." Fruit wine not derived wholly from one kind of fruit shall be designated as "fruit wine" or "berry wine," as the case may be, qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Fruit wines which are derived wholly (except for sugar, water, or added alcohol) from apples or pears may be designated "cider" and "perry," respectively, and shall be so designated if lacking in vinous taste, aroma, and characteristics. Fruit wine rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle shall be further designated as "sparkling"; and fruit wine rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated."

Class 6. *Wine from other agricultural products.*—(*a*) Wine of this class is wine (other than grape wine, citrus wine, or fruit wine) made by the normal alcoholic fermentation of sound fermentable agricultural products, either fresh or dried, or of the restored or unrestored pure condensed must thereof, with the addition before or during fermentation of a volume of water not greater than the minimum necessary to correct natural moisture deficiencies in such products, with or without the addition, after fermentation, of pure condensed must, and with or without added alcohol or such other fortifying spirits as will not alter the character of the product, but without other addition or abstraction except as may occur in cellar treatment, *Provided*, That the product may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent, but in no event shall any product so ameliorated have an alcoholic content, derived by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural wine of this class, more than 0.14 gram, and for other wine of this class, more than 0.12 gram, per 100 cubic centimeters (20°C.).

(*b*) "Light" wine of this class is wine having an alcoholic content not in excess of 14 per cent by volume.

(*c*) "Natural" wine of this class is wine containing no fortifying spirits or added alcohol.

(*d*) "Raisin wine" is wine of this class made from dried grapes.

(*e*) "Sake" is wine of this class produced from rice in accordance with the commonly accepted method of manufacture of such product.

(*f*) Wine of this class derived wholly (except for sugar, water, or added alcohol) from one kind of agricultural product shall, except in the case of "sake," be designated by the word "wine" qualified by the name of such agricultural product, *e.g.*, "honey wine," "raisin wine," "dried blackberry wine." Wine of this

class not derived wholly from one kind of agricultural product shall be designated as "wine" qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Wine of this class rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of wine within a closed container, tank, or bottle shall be further designated as "sparkling"; and wine of this class rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated."

Class 7. *Vermouth.*—(*a*) "Vermouth" is a compound having an alcoholic content of not less than 15 per cent by volume, made by the mixture of extracts from macerated aromatic flavoring materials with grape wine containing fortifying grape spirits or added alcohol, and manufactured in such a manner that the product possesses the taste, aroma, and characteristics generally attributed to vermouth.

Class 8. *Imitation, concentrate, and substandard wine.*—(*a*) "Imitation wine" shall bear as a part of its designation the word "imitation," and shall include:

(1) Any wine containing synthetic materials.

(2) Any wine made from a mixture of water with residue remaining after thorough pressing of grapes, fruit, or other agricultural products.

(3) Any class or type of wine the taste, aroma, color, or other characteristics of which have been acquired in whole or in part, by treatment with methods or materials of any kind, if the taste, aroma, color, or other characteristics of normal wines of such class or type are acquired without such treatment.

(*b*) "Concentrate wine" shall bear as a part of its designation the word "concentrate," and shall include any wine made from must concentrated at any time to more than 80° (Balling).

(*c*) "Substandard wine" shall bear as a part of its designation the word "substandard," and shall include:

(1) Any wine having a volatile acidity in excess of the maximum prescribed therefor in this article.

(2) Any wine for which no maximum volatile acidity is prescribed in this article, having a volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, in excess of 0.14 gram per 100 cubic centimeters (20°C.).

(3) Any wine for which a standard of identity is prescribed in this article, which, through disease, decomposition, or otherwise, fails to have the composition, color, and clean vinous taste and aroma of normal wines conforming to such standard.

Sec. 22. *Blends, cellar treatment, alteration of class or type.*—(*a*) If the class or type of any wine shall be altered, and if the product as so altered does not fall within any other class or type either specified in this article or known to the trade, then such wine shall, unless otherwise specified in this section, be designated with a truthful and adequate statement of composition in accordance with section 34, article III, of these regulations.

(*b*) Alteration of class or type shall be deemed to result from any of the following occurring before, during, or after production:

(1) Treatment of any class or type of wine with substances foreign to such wine which remain therein, *Provided,* That the presence in finished wine of not

more than 350 parts per million (not more than 70 of such parts being in a free state) of total sulphur dioxide, or sulphites expressed as sulphur dioxide, shall not be precluded under this paragraph.

(2) Treatment of any class or type of wine with substances not foreign to such wine but which remain therein in larger quantities than are naturally and normally present in other wines of the same class or type not so treated.

(3) Treatment of any class or type of wine with methods or materials of any kind to such an extent or in such manner as to affect the basic composition of the wine so treated by altering any of its characteristic elements.

(4) Blending of wine of one class with wine of another class or the blending of wines of different types within the same class.

(5) Treatment of any class or type of wine for which a standard of identity is prescribed in this article with sugar or water in excess of the quantities specifically authorized by such standard, *Provided,* That where such wine is derived exclusively from fruit or other agricultural products the normal acidity of which is 20 parts or more per thousand, and such wine has been manufactured in accordance with the standard of identity therefor in all respects except that the volume of the product has been increased more than 35 per cent, but not more than 60 per cent, by the addition of sugar and water solution for the sole purpose of correcting natural deficiencies due to such acidity, the class or type shall not be deemed to be altered but there shall be stated, as a part of the class or type designation, the phrase "made with over 35 per cent sugar solution."

(*c*) Nothing in this section shall preclude the treatment of wine of any class or type in the manner hereinafter specified, provided such treatment does not result in the alteration of the class or type of the wine under the provisions of paragraph (*b*) of this section.

(1) Treatment with filtering equipment, and with fining or sterilizing agents.

(2) Treatment with pasteurization at the minimum temperature and for the minimum period necessary to accomplish practical stabilization, but not for the purpose of shortening the normal maturation period.

(3) Treatment with refrigeration at the maximum temperature and for the minimum period necessary to accomplish practical stabilization, but not for the purpose of shortening the normal maturation period.

(4) Treatment with methods and materials to the minimum extent necessary to correct cloudiness, precipitation, or abnormal color, odor, or flavor developing in wine.

(5) Treatment with constituents naturally present in the kind of fruit or other agricultural product from which the wine is produced for the purpose of correcting deficiencies of these constituents, but only to the extent that such constituents would be present in normal wines of the same class or type not so treated.

Sec. 23. *Grape type designations.*—(*a*) A name indicative of a variety of grape may be employed as the type designation of a grape wine if the wine derives its predominant taste, aroma, and characteristics, and at least 51 per cent of its volume, from that variety of grape. If such type designation is not known to the consumer as the name of a grape variety, there shall appear in direct connection therewith an explanatory statement as to the significance thereof.

Sec. 24. *Generic, semi-generic, and non-generic designations of geographic significance.*—(*a*) A name of geographic significance which is also the designation of a class or type of wine, shall be deemed to have become generic only if so found by the Administrator.

Examples of generic names, originally having geographic significance, which are designations for a class or type of wine are: vermouth, sake.

(*b*) A name of geographic significance, which is also the designation of a class or type of wine, shall be deemed to have become semi-generic only if so found by the Administrator. Semi-generic designations may be used to designate wines of an origin other than that indicated by such name only if there appears in direct conjunction therewith an appropriate appellation of origin disclosing the true place of origin of the wine.

Examples of semi-generic names which are also designations for types of grape wine are: Angelica, Burgundy, Claret, Chablis, Champagne, Chianti, Malaga, Marsala, Madeira, Moselle, Port, Rhine Wine (syn. Hock), Sauterne, Haut Sauterne, Sherry, Tokay.

(*c*) A name of geographic significance, which has not been found by the Administrator to be generic or semi-generic, may be used only to designate wines of the origin indicated by such name, but such name shall not be deemed to be the distinctive designation of a wine unless the Administrator finds that it is known to the consumer and to the trade as the designation of a specific wine of a particular place or region, distinguishable from all other wines.

Examples of non-generic names which are not distinctive designations of specific wines are: American, California, Lake Erie Islands, Napa Valley, New York State, French, Spanish.

Examples of non-generic names which are also distinctive designations of specific grape wines are: Bordeaux Blanc, Bordeaux Rouge, Graves, Medoc, St. Julien, Chateau Yquem, Chateau Margaux, Chateau Lafite, Pommard, Chambertin, Montrachet, Rhone, Liebfraumilch, Rudesheimer, Forster Deidesheimer, Schloss Johannisberger, Lagrima, Lacryma Christi.

Sec. 25. *Appellations of origin.*—(*a*) A wine shall be entitled to an appellation of origin if (1) at least 75 per cent of its volume is derived from fruit or other agricultural products both grown and fermented in the place or region indicated by such appellation, (2) it has been fully manufactured and finished within such place or region, and (3) it conforms to the requirements of the laws and regulations of such place or region governing the composition, method of manufacture, and designation of wines for home consumption.

(*b*) Wines subjected to cellar treatment outside the place or region of origin under the provisions of section 22(*c*) of this article, and blends of wines of the same origin blended together outside the place or region of origin (if all the wines in the blend have a common class, type, or other designation which is employed as the designation of the blend), shall be entitled to the same appellation of origin to which they would be entitled if such cellar treatment or blending took place within the place or region of origin.

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CHAPTER VII

THE DISTILLING INDUSTRIES

The distilling industries are those concerned with the production of distilled spirits: rum, whisky, brandy, gin, and cordials and liqueurs.

Production Statistics.—Table 27 shows the production of distilled spirits for the fiscal year 1938, ended June 30, 1938.

TABLE 27.—PRODUCTION OF DISTILLED SPIRITS, FISCAL YEAR 1938[1]
(Tax gallons)

State	Whisky	Rum	Gin[2]	Brandy	Other spirits	Total
Arizona	149,111	....			1,806	150,917
California	1,595,623	....	287,493	21,233,789	15,638	23,132,543
Connecticut				61,174		61,174
Hawaii				21,696		21,696
Illinois	29,261,867		2,091,559		759,122	,112,548
Indiana	9,667,981		955,819		10,240,061	,863,861
Kentucky	41,671,416	865,166				,536,582
Louisiana	300,640	186,587	8,393			495,620
Maryland	10,970,807			12,444		,983,251
Massachusetts	2,105,519	1,012,400				,117,919
Michigan	100,166					100,166
Missouri	346,142					346,142
Montana	53,811					53,811
New Jersey				465,281		465,281
New York			1,160,691	337,805	3,306,596	,805,092
Ohio	1,418,520					,418,520
Oregon				92,929		92,929
Pennsylvania	4,674,504	106,116	1,644,045	72,855	2,074,230	,571,750
Virginia	551,087			76,370		627,457
Washington				169,987		169,987
Wisconsin	28,678					28,678
Total	102,895,872	2,170,26	6,148,000	22,544,330	16,397,453†	150,155,924

[1] U.S. Dept. of the Treasury, Bureau of Internal Revenue, Alcohol Tax Unit, Nov. 21, 1938.
[2] For additional production of gin at rectifying plants see Table 14 of reference quoted.
* Includes 1,448,370 gal. produced at distilleries for denaturation.
† All high-proof spirits produced under provisions of the Liquor Tax Administration Act of June 26, 1936, except 136,666 gal. of spirits produced for denaturation and 1,806 gal. of tequila.

An examination of this table shows that Kentucky, Illinois, Maryland, and Indiana led the country in the production of whisky (in the order named), while rum was produced principally in Massachusetts and Kentucky.

Illinois led the United States in the production of gin, with Pennsylvania, New York, and Indiana following in the order named.

California was outstandingly the leader in brandy manufacture, while Indiana led in the production of other spirits.

RUM

"Rum" is any alcoholic distillate from the fermented juice of sugarcane, sugarcane sirup, sugarcane molasses, or other sugarcane by-products distilled at less than 190° proof (whether or not such proof is further reduced prior to bottling

TABLE 28.—ANALYSES OF SOME RUMS[1]
(Grams per 100 liters not calculated to proof)

Proof	pH	Total acid (as acetic)	Volatile acid (as acetic)	Esters (as ethyl acetate)	Fusel oil (as amyl alcohol)	Solids	Aldehydes	Furfural	Color in 0.5-in. cell	Source, comments
103.0	4.76	9.6	9.6	16.7	123.2	8	4.4	0.0	0.2	Pennsylvania, new
103.6	4.54	33.6	26.4	23.8	124.3	48	4.7	Trace	4.0	Pennsylvania, 0.5 year old
104.8	4.52	36.0	28.8	26.4	124.0	50	5.5	0.8	4.5	Pennsylvania, 1 year old
107.8	4.44	50.4	40.8	33.4	132.0	94	6.8	0.8	7.0	Pennsylvania, 2 years old
102.0	4.28	14.4	14.4	43.1	102.1	16	40	5.5	0.0	Kentucky, new
104.4	4.17	80.4	67.2	60.7	108.6	168	40	8.2	13.5	Kentucky, 1 year old
106.8	4.28	88.8	73.2	73.0	116.2	204	34.3	12.0	17.0	Kentucky, 2 years old
100.6	4.68	38.4	36.0	22.0	91.7	6	4.0	1.2	0.0	Massachusetts, new
100.4	4.42	84.0	74.0	29.0	91.5	86	6.4	1.2	7.5	Massachusetts, 0.5 year old
101.4	4.37	93.4	86.4	45.5	93.3	142	7.2	1.4	11.0	Massachusetts, 1.5 years old
102.4	4.30	98.4	86.4	51.0	96.8	162	7.2	1.6	13.0	Massachusetts, 2 years old
135.2	4.07	184.8	148.8	216.0	450.6	274	48	7.2	18.0	Old New England, approx. 19 years old
87.3	5.03	13.2	12.0	11.4	49.3	678	1.6	0.1	14.5, caramel	Cuba, Ron Bacardi Superior, gold
92.8	8.05	4.8	2.4	44.0	59.8	205	7.0	0.8	15.5	St. Croix cane juices, 2 years old
98.6	4.17	57.6	48.0	59.8	91.5	450	20.0	4.0	23.0, caramel	Jamaica, 15 years old
92.8	4.32	62.4	43.2	26.4	68.6	1,510	0.8	0.0	34.0, caramel	Demerara

[1] VALAER, P., Foreign and Domestic Rum, *Ind. Eng. Chem.*, **29**: 988 (1937).

to not less than 80° proof) in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to rum; and includes mixtures solely of such distillates.[1]

Rum is manufactured in general in those countries which grow sugarcane or import molasses or other sugarcane products. It is made principally in the United States, Puerto Rico, Cuba, Jamaica, St. Thomas and

[1] Dept. of the U.S. Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939.

St. Croix (Virgin Islands of the United States), British Virgin Islands, Demerara, Barbados, Uruguay, Martinique, Trinidad, Haiti, Santo Domingo, the Leeward Islands, Guadelupe, Mexico, Grenada, and Antigua.

Composition.—Table 28 gives analyses of some rums from different sources.

Acids (volatile and nonvolatile), esters, fusel oil, and aldehydes are regular constitutents of rum. Ethyl acetate is the most common ester produced, while both acetaldehyde and furfural ($C_4H_3O \cdot CHO$) are always found, in traces at least, in rum.

Types.—Rums made in the United States, with certain exceptions, may be classified as heavy-bodied rums.[1] They contain only the congenerics (acids, esters, etc.) that are accumulated during distillation and aging in charred white oak barrels. Most of the rums are distilled at a proof less than 160° and aged for several years. They contain no coloring matter added by the distillers.

Rums produced outside the United States, with exceptions, vary from very light bodied to very heavy bodied. Cuba produces some of lightest bodied rums, while Jamaica manufactures the heaviest bodied rums outside the United States. Substances are frequently added to such rums to bring about the desired characteristics, one of these substances being caramel for artificial color (not all countries permit these additions, however).

Table 29 gives the analyses of some light-, medium-, and heavy-bodied rums.

Production of Rum. *Fermentation.*—The process for the manufacture of rum is similar in many respects to that in which industrial alcohol is produced. Methods for the propagation of pure cultures of yeast, the building up of a starter, the reaction of the main mash, the concentration of the fermentable sugar, the temperature of the fermentation, and even the principal end products are similar. Some rums (Jamaica) are made by spontaneous fermentation, however.

In the manufacture of rum, the careful selection of the raw materials, the control of the fermentation, proper distillation, and the aging of the distilled product are essential for the production of a fine rum.

Strains of *Saccharomyces cerevisiae* or of other yeasts, such as species of *Schizosaccharomyces*, may be used to pitch (inoculate) the mash. Great care must be exercised to maintain the selected yeast in pure culture, if the fermentation is to be successful.

As already indicated, rum is prepared from sugar-cane products. In the United States only blackstrap molasses is used in rum manufacture. This substance can be purchased at relatively low cost when procured

[1] VALAER, P., Foreign and Domestic Rum, *Ind. Eng. Chem.*, **29**: 988 (1937).

in large quantities. It is a product of the sugar mill after the crystallizable sugar has been removed, and contains 40 to 55 per cent of fermentable sugar usually.

TABLE 29.—ANALYSES OF SOME LIGHT-, MEDIUM-, AND HEAVY-BODIED RUMS[1]

Type of rum	Manufacture and location	Grams per 100 liters of 100 proof								
		Proof	Total acid (as acetic)	Esters (as ethyl acetate)	Aldehyde	Furfural	Fusel oil (as amyl alcohol)	Color	Wood extract	
Light bodied	Bacardí (Cuba), Carta Blanca[2]	89.6	9.1	11.7	5.4	0.1	65.0	Light	Slight	Bottled for sale in the United States
	Castillo (Cuba), Carta Oriente[3]	87.8	9.7	7.0	7.3	0.1	79.0	Dark	Slight	Bottled for sale in the United States
Medium bodied	Ron Rico (Puerto Rico), White Label[4]	82.9	28.	32.	19.	0.6	136.	Light	Present	Bottled for sale in the United States
	Bacardí (Cuba), "Extra Superior, 1873"[5]	90.1	53.	25.	11.	0.8	80.	Dark	Present	Bottled for sale in the United States
Heavy bodied	Wray's 3 Dagger (Jamaica)[6]	99.8	34.0	56.0	18.0	4.0	238.	Dark	Present	For the United States trade. Bottled sample
	(Jamaica) for sale to Germany[7]	149.	141.	565.	19.0	5.4	114.	Dark	Present	High-proof. From a barreled sample

[1] Courtesy of Dr. Walter C. Tobie.
[2] Typical of a very light-flavored Cuban rum, such as is frequently used for highballs and cocktails.
[3] Although the color is dark, the flavor of this rum is light.
[4] An early sample (about 1934) for shipment to the United States.
[5] This is one of the heaviest Cuban rums. The flavor is relatively rich and fruity.
[6] A typical Jamaica rum for sale in the United States. The flavor is high.
[7] Bulk rum, said to have been prepared in Jamaica for the German trade. Note the excessively high ester value.

A concentration of molasses that contains from 12 to 14 per cent of fermentable sugars may be used in preparing the main mashes. Ammonium sulphate, and occasionally phosphates, may be added as a source of food for the yeast. Dunder, *i.e.*, distillation slops which may be decomposed, may be used to give the rum a heavier flavor and to supply nutrient material.

Sulphuric acid is used to adjust the pH of the mash to 4.0 to 4.7, the latter pH favoring a rapid fermentation and the production of a light-flavored rum. The use of lower pH values favors slower fermentations and the production of heavy-bodied rums, such as the Jamaica type. It is usually necessary to add acid to the mash, unless it has been heat sterilized, to inhibit the development of undesirable bacteria.

The fermentation tanks must be cleaned carefully after being used if a clean fermentation and light-bodied rum are desired. Steam, sulphuric acid, ammonium bifluoride, or other agents may be used to prevent the development of undesirable flora in the tank.

A fermentation temperature of about 70°F. (initial) to 96°F. (final) is satisfactory. Low temperatures favor a slow fermentation, while at high temperatures volatile constituents are lost.

Although the main fermentation is nearly complete within 2 days, the mashes are permitted to ferment for 3 to 7 days before being distilled. A 6-day or 144-hr. fermentation period is used in at least one modern distillery in this country.

The fermented mash is sometimes designated as "beer."

Distillation.—Distillation is an important process in the production of rum. Mashes that are distilled at high proof produce light-flavored rums, which are expected to be consumed shortly. Only small changes take place in such products during storage, for the rums contain but small quantities of acids, esters, fusel oil, and other congeneric substances. As stated earlier, most rums in this country are distilled at a proof of less than 160°.

Various types of distillation equipment are used: pot stills, continuous-type stills with combined rectifying columns, beer stills with doublers, chambered stills, and other modifications.

In the United States, rum distilled at a low proof is usually adjusted to 100° proof before being aged.

Aging.—Aging is a process for improving the character of rum, for decreasing the undesirable flavors, and for greatly increasing the pleasant ones. Flavor, color, and aroma are developed, while the product is mellowed. During the first 6 months of the process, the main increases in esters, acids, solids, and color take place. In a freshly distilled rum, the quantity of esters usually exceeds that of the acids, but after storage for less than 6 months the ratio changes. Indeed, the acid content is usually greater than the ester content after two years,[1] owing largely to the acidic products extracted from the wood of the barrel.

Rum is usually aged in charred white oak barrels, although some is aged in plain cooperage. The aging is permitted to proceed for a few

:, *loc. cit.*

months or for several years (in Government bonded warehouses in the United States).

Some rum is "quick-aged" by placing charred white oak chips in the rum before it is stored. Quick-aging may also be accomplished by circulating the rum over oak chips. Heat and aeration are sometimes used. Quick-aging processes are not particularly satisfactory, however.

Government Supervision.—The manufacture of rum in the United States and its possessions is carried out under Government supervision. The quantity of raw material used and rum produced are carefully measured. The Bureau of Internal Revenue is interested in taxes, and the Federal Alcohol Administration in the promulgation of regulations concerning standards for identification, such as labeling and advertising.

Labeling.—According to the Federal Alcohol Administration Division,[1] a bottled rum should contain the following information on the label: the class and type, the alcoholic content, the net contents, and the presence of "artificial or excessive coloring or flavoring." A statement concerning the age of the rum may also be placed on the label, if desired.

Uses.—Rum may be used in the preparation of ice cream, candies, and mincemeat; in the curing of tobacco; as a beverage; and as a medicinal.

Definitions and Regulations.—"New England rum" is rum that is produced in the United States, is distilled at less than 160° proof and is a straight rum and not a mixture of rums.[2]

Puerto Rico, Cuba, Demerara, Barbados, St. Croix, St. Thomas, Virgin Islands, Jamaica, Martinique, Trinidad, Haiti, and San Domingo rum are not distinctive types of rum. Such names are not generic but retain their geographic significance. They may not be applied to rum produced in any other place than the particular region indicated in the name, and may not be used as a designation of a product as rum, unless such product is rum as defined [see page 135].

Imitation rum—(1) neutral spirits or other distilled spirits which have been added thereto or which contain synthetic or imitation rum flavoring materials, and (2) rum which has added thereto neutral spirits or other distilled spirits than rum are "imitation rum" and shall be so designated.

WHISKY

According to Article II, Section 21, Class 2 of the Federal Alcohol Administration Act, Regulations 5,[2]

"Whisky" is an alcohol distillate from a fermented mash of grain distilled at less than 190° proof in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to whisky, and withdrawn from

[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939.

the cistern room of the distillery at not more than 110° proof and not less than 80° proof, whether or not such proof is further reduced prior to bottling to not less than 80° proof; and also includes mixtures of the foregoing distillates for which no specific standards of identity are prescribed herein. . . .

Types.—There are several types of whisky. Standards for the identity of American types and some others are stated later in this chapter.

Composition of Whiskies.—Whiskies contain approximately 50 per cent by volume ethyl alcohol. The flavor and bouquet are due mainly to the impurities or congeneric substances that they contain, however. These accumulate during the production and storage of the whisky.

In whisky are found acids, esters, aldehydes, furfural, fusel oil, and solids, in addition to ethyl alcohol and water. The principal acids are acetic and valeric acids,[1] but traces of propionic and other acids are found also. The combination of ethyl alcohol with these acids yields ethyl acetate, ethyl valerate, and ethyl propionate, respectively, while the amyl alcohols present in the fusel oil form amyl acetate, amyl valerate, and other esters. Aldehydes include acetaldehyde and those resulting from the oxidation of small amounts of some of the higher alcohols during aging. Fusel oil is composed mainly of higher alcohols.

In Table 30 are given the average analyses for 31 different samples (barrels) of whisky, of which 14 were rye whisky, 13 being prepared by the sweet-mash and 1 by the sour-mash method, and of which 17 were bourbon whisky, 13 being prepared by the sour-mash and 4 by the sweet-mash method. The average data for the rye and bourbon whiskies are also given. The effect of aging on the quantities of the various components of whisky is illustrated. The results are expressed in grams per 100 liters, calculated to the original volume of the whisky. The acids are calculated as acetic acid, the esters and aldehydes as acetic, the fusel oil as amyl alcohol.

Production of Whisky.—The flavor, aroma, and characteristics of the final product depend on the nature of the raw materials and the fermentation, the method of distillation, and the aging process.

1. *Raw Materials.*—Grains are the raw materials from which whiskies are produced, the usual ones being rye, corn, wheat, and barley.

In the United States, rye and bourbon are the principal types of whisky manufactured. Rye whisky is generally prepared from rye and rye malt or from rye and barley malt. A typical mash may contain 80 per cent rye and 20 per cent barley malt. At least 51 per cent rye is required by law. Bourbon, on the other hand, is prepared from corn (maize or Indian), barley malt or wheat malt, and usually another grain.

[1] WOODMAN, A. G., "Food Analysis," 3d ed., McGraw-Hill Book Company, Inc., 1931.

A typical mash may contain, for example, 70 per cent corn, 15 per cent rye and 15 per cent malt, or 65 per cent corn, 23 per cent rye and 12 per cent malt. The mash must always contain at least 51 per cent corn (see the standards of identity for whisky given in this section).

TABLE 30.—AVERAGE ANALYSES OF SOME WHISKIES[1]
(Calculated to original volume)

Age, years	Type	Color	Solids	Acids	Esters	Aldehydes	Furfural	Fusel oil
	Whole	....	20.0	6.4	15.0	4.03	0.71	96.8
New	Rye	....	13.6	4.7	13.7	4.91	0.97	83.2
	Bourbon	....	26.0	7.7	17.2	3.26	0.44	108.6
	Whole	7.3	101.5	37.8	29.9	7.08	1.5	106.2
1	Rye	8.4	114.6	41.8	35.3	8.71	1.7	106.8
	Bourbon	6.4	90.1	34.4	24.9	5.55	1.3	105.8
	Whole	8.6	124.2	46.1	42.9	8.34	1.7	108.1
2	Rye	10.6	133.6	49.8	49.3	9.02	1.9	109.7
	Bourbon	6.7	114.8	42.7	37.3	7.78	1.4	107.3
	Whole	10.2	140.2	51.1	48.4	9.47	1.8	106.3
3	Rye	11.5	150.4	54.4	54.3	9.80	2.2	104.4
	Bourbon	8.3	130.7	47.8	42.5	9.15	1.5	107.3
	Whole	10.2	140.4	51.6	50.9	10.2	1.9	104.3
4	Rye	11.6	153.1	54.2	57.2	11.2	2.2	102.0
	Bourbon	8.9	127.7	48.9	45.0	9.3	1.5	106.3
	Whole	11.1	149.2	52.2	51.1	10.2	1.9	100.4
5	Rye	12.2	158.8	54.8	57.5	11.3	2.5	100.1
	Bourbon	10.0	140.2	49.8	45.0	9.2	1.5	100.7
	Whole	11.1	151.4	53.2	50.7	10.2	1.9	104.7
6	Rye	12.3	161.0	54.8	55.5	11.3	2.4	105.9
	Bourbon	10.1	142.5	51.8	45.2	9.1	1.4	103.8
	Whole	11.1	154.0	52.2	51.1	9.8	1.8	99.9
7	Rye	12.0	161.3	51.9	56.6	10.6	2.2	98.8
	Bourbon	10.2	147.1	52.4	46.4	9.0	1.4	101.6
	Whole	10.5	155.2	53.1	50.9	9.6	1.8	98.0
8	Rye	11.1	163.8	52.6	56.7	10.6	2.2	99.0
	Bourbon	10.0	147.7	53.6	45.9	8.8	1.5	97.1

[1], C. A., and L. M. TOLMAN, *Jour. Am. Chem. Soc.*, **30**: 98 (1908).

The preparation of a mash for whisky is similar in fundamental details to the preparation of a mash in the brewing industry, except

that the solids are not removed (Chap. V). The enzymes of the malt convert the starches of the cooked grains to dextrins and sugars. At the same time, the protein molecules are degraded sufficiently to furnish the yeast with a satisfactory source of nitrogen.

2. *Fermentation.*—The resultant mash is fermented by a strain of distiller's yeast, *Saccharomyces cerevisiae*, by either the sweet- or sour-mash method. In the former method, which is commonly used, the mash is inoculated directly with the yeast. Less time is required for this fermentation, generally, and a higher yield of alcohol is obtained than from fermented sour mashes. In the latter method the spent slops and barm (yeast) from tanks previously set and fermented are mixed with the mash before fermentation.

Bacteria play a part in the production of esters in whisky manufacture, the temperatures attained during fermentation favoring their growth. Not infrequently the distiller may grow his selected culture of yeast in a medium containing lactic acid bacteria, the lactic acid favoring the growth of yeast, inhibiting certain undesirable types of microorganisms, and contributing to the aroma, flavor, and characteristics of the whisky.

3. *Distillation.*—Both the type of still and the method of distillation are important, for they contribute to the quality of the product. The continuous still, with doubler, and the three-chambered still, with doubler, are two commonly used stills.

4. *Aging.*—During the process of aging, definite changes take place in certain of the congeneric substances of whisky as the result of storing it in oak containers. These changes have been investigated in great detail by Crampton and Tolman (1908) and by Valaer and Frazier (1936).

"Age" for "American type whiskeys, other than corn whiskey, straight corn whiskey, blended corn whiskey, and blends of straight corn whiskey, produced on or after July 1, 1936, means the period during which the whiskey has been kept in charred new oak containers."[1]

Whisky is stored in heated or unheated warehouses for aging, heat causing greater extraction of substances from the wood of the container.

CHANGES DURING AGING.—During aging in white oak barrels, changes take place in the solids, esters, acids, fusel oil, aldehydes, furfural, and color, the largest increase in solids, esters, acids, and color occurring during the first half year of storage.[2]

The solids of whisky, known also as extract, are derived from the wood of the container. During the first 6 months of storage, Valaer

[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, as amended to Mar. 1, 1939.

[2] VALAER, P., and W. H. FRAZIER, *Ind. Eng. Chem.*, **28**: 92 (1936).

and Frazier found an average increase of about 70 g. of solids per 100 liters. The quantity of extract was progressively smaller during each succeeding 6-month period, being apparently 4 g. per 100 liters during the period between the forty-second and forty-eighth months of storage.

Since there is very little change in the ester content of a whisky during quick-aging, the determination of esters may be used as a dependable index of the age of the whisky.

The acids of whisky are both volatile and fixed acids. The larger part of the increase in total acids during storage is due to volatile acids. Some fixed acids are extracted from the wood of the container.

During storage in charred oak barrels, there is an actual loss in the quantity of fusel oil, according to Valaer and Frazier. The fusel-oil determination gives information concerning the method of distillation and is, therefore, considered to be an important determination.

There is an actual loss of aldehydes during storage.

Some furfural is extracted from charred oak barrels during the first 6 months of storage, but very little appears later. Uncharred barrels do not contribute furfural.

QUICK-AGING.—The process of quick-aging of whisky is used extensively, especially for whiskies that are to be marketed shortly after manufacture. Quick-aging produces but little change in the ester content and no effect on the fusel oil. It increases the aldehydes only slightly. Solids, acids (mainly the nonvolatile ones), furfural, and color are increased. Color depth is readily varied by means of this process. The following table illustrates the effect of quick-aging on a whisky.

TABLE 31.—DIFFERENCE IN SAME WHISKY WITH AND WITHOUT QUICK-AGING[1]

	Grams per 100 liters calculated to proof	
	Quick-aged	Not quick-aged
Total acids	11.9	5.9
Esters	14.3	15.6
Fusel oil	124.3	127.6
Solids	33.6	11.2
Color	3.5	0.0
Furfural	1.0	0.0

[1] VALAER, P., and W. H. FRAZIER, *Ind. Eng. Chem.*, **28**: 92 (1936).

Heat, charred or uncharred wood chips, and charred barrels are used in some of the quick-aging processes.

CHANGES IN GLASS.—Although extensive changes do not usually occur in whiskys stored in glass containers, evidence indicates that there may be a slight decrease in acids in the case of some whiskies, especially those which are not new. Valaer and Frazier suggest that this reduction in acidity may be due to the alkali dissolved from the glass, to the establishment of a change in equilibrium, or to an oxidation change. Esters tend to increase as does the color, while furfural is reduced in quantity.

Federal Control.—The manufacture of whisky is carried out under Government supervision from the weighing of the grain to the sale of the whisky. The Bureau of Internal Revenue and the Federal Alcohol Administration Division are much interested in the control of the whisky production. For details in connection with importing, bottling, labeling, and advertising, the reader is referred to the publications of the latter division, some of which are listed in the references given at the end of this section.

Standards of Identity.—Standards of identity for different types of whiskies, under the provisions of the Federal Alcohol Administration Act, are as follows ("American type" whiskies being specified in subsections (*a*) through (*j*)):

(*a*) "Rye whiskey," "bourbon whiskey," "wheat whiskey," "malt whiskey," or "rye malt whiskey" is whiskey which has been distilled at not exceeding 160° proof from a fermented mash of not less than 51% rye grain, corn grain, wheat grain, malted barley grain or malted rye grain, respectively, and, if produced on or after March 1, 1938, stored in charred new oak containers, and also includes mixtures of such whiskeys where the mixture consists exclusively of whiskeys of the same type. "Corn whiskey" is whiskey which has been distilled at not exceeding 160° proof from a fermented mash of not less than 80% corn grain, stored in uncharred oak containers or reused charred oak containers, and not subjected, in the process of distillation or otherwise, to treatment with charred wood, and also includes mixtures of such whiskey.

(*b*) "Straight whiskey" is an alcoholic distillate from a fermented mash of grain distilled at not exceeding 160° proof and withdrawn from the cistern room of the distillery at not more than 110° and not less than 80° proof, whether or not such proof is further reduced prior to bottling to not less than 80° proof, and is—

(1) Aged for not less than twelve calendar months if bottled on or after July 1, 1936, and before July 1, 1937; or

(2) Aged for not less than eighteen calendar months if bottled on or after July 1, 1937, and before July 1, 1938; or

(3) Aged for not less than twenty-four calendar months if bottled on or after July 1, 1938.

The term "straight whiskey" also includes mixtures of straight whiskey which, by reason of being homogeneous, are not subject to the rectification tax under the Internal Revenue Laws.

(*c*) "Straight rye whiskey" is straight whiskey distilled from a fermented mash of grain of which not less than 51% is rye grain.

(*d*) (1) "Straight bourbon whiskey" is straight whiskey distilled[1] from a fermented mash of grain of which not less than 51% is corn grain.

(2) "Straight corn whiskey" is straight whiskey distilled from a fermented mash of grain of which not less than 80% is corn grain, aged for the required period in uncharred oak containers or reused charred oak containers, and not subjected, in the process of distillation or otherwise, to treatment with charred wood.

(*e*) "Straight wheat whiskey" is straight whiskey distilled from a fermented mash of grain of which not less than 51% is wheat grain.

(*f*) "Straight malt whiskey" and "straight rye malt whiskey" are straight whiskey distilled from a fermented mash of grain of which not less than 51% of the grain is malted barley or malted rye, respectively.

(*g*) "Blended whiskey" (whiskey—a blend) is a mixture which contains at least 20% by volume of 100° proof straight whiskey and, separately or in combination, whiskey or neutral spirits, if such mixture at the time of bottling is not less than 80° proof.

(*h*) "Blended rye whiskey" (rye whiskey—a blend), "blended bourbon whiskey" (bourbon whiskey—a blend), "blended corn whiskey" (corn whiskey—a blend), "blended wheat whiskey" (wheat whiskey—a blend), "blended malt whiskey" (malt whiskey—a blend) or "blended rye malt whiskey" (rye malt whiskey—a blend) is blended whiskey which contains not less than 51% by volume of straight rye whiskey, straight bourbon whiskey, straight corn whiskey, straight wheat whiskey, straight malt whiskey, or straight rye malt whiskey, respectively.

(*i*) "A blend of straight whiskeys" (blended straight whiskeys), "a blend of straight rye whiskeys" (blended straight rye whiskeys), "a blend of straight bourbon whiskeys" (blended straight bourbon whiskeys), "a blend of straight corn whiskeys" (blended straight corn whiskeys), "a blend of straight wheat whiskeys" (blended straight wheat whiskeys), "a blend of straight malt whiskeys" (blended straight malt whiskeys), and "a blend of straight rye malt whiskeys" (blended straight rye malt whiskeys) are mixtures of only straight whiskeys, straight rye whiskeys, straight bourbon whiskeys, straight corn whiskeys, straight wheat whiskeys, straight malt whiskeys, or straight rye malt whiskeys, respectively.

(*j*) "Spirit whiskey" is a mixture (1) of neutral spirits and not less than 5% by volume of whiskey, or (2) of neutral spirits and less than 20% by volume of straight whiskey, but not less than 5% by volume of straight whiskey, or of straight whiskey and whiskey, if the resulting product at the time of bottling be not less than 80° proof.

(*k*) "Scotch whiskey" is a distinctive product of Scotland, manufactured in Scotland in compliance with the laws of Great Britain regulating the manufacture of Scotch whiskey for consumption in Great Britain, and containing no distilled spirits less than three years old: *Provided*, That if in fact such product as so manufactured is a mixture of distilled spirits, such mixture is "blended Scotch

[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Amendment 5 to Regulations 5.

whiskey" (Scotch whiskey—a blend). "Scotch whiskey" shall not be designated as "straight."

(*l*) "Irish whiskey" is a distinctive product of Ireland, manufactured either in the Irish Free State or in Northern Ireland, in compliance with the laws of those respective territories regulating the manufacture of Irish whiskey for consumption in such territories, and containing no distilled spirits less than three years old: *Provided*, That if in fact such product as so manufactured is a mixture of distilled spirits, such whiskey is "blended Irish whiskey" (Irish whiskey—a blend). "Irish whiskey" shall not be designated as "straight."

(*m*) "Canadian whiskey" is a distinctive product of Canada, manufactured in Canada in compliance with the laws of the Dominion of Canada regulating the manufacture of whiskey for consumption in Canada, and containing no distilled spirits less than two years old: *Provided*, That if in fact such product as so manufactured is a mixture of distilled spirits, such whiskey is "blended Canadian whiskey" (Canadian whiskey—a blend). "Canadian whiskey" shall not be designated as "straight."

(*n*) "Blended Scotch type whiskey" (Scotch type whiskey—a blend) is a mixture made outside Great Britain and composed of—

(1) Not less than 20% by volume of 100° proof malt whiskey or whiskeys distilled in pot stills at not more than 160° proof, from a fermented mash of malted barley dried over peat fire, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof, and

(2) Not more than 80% by volume of neutral spirits, or whiskey distilled at more than 180° proof, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof.

(*o*) "Blended Irish type whiskey" (Irish type whiskey—a blend) is a product made outside Great Britain or the Irish Free State and composed of—

(1) A mixture of distilled spirits distilled in pot stills at not more than 171° proof, from a fermented mash of small cereal grains of which not less than 50% is dried malted barley, and unmalted barley, wheat, oats, or rye grains, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof; or

(2) A mixture consisting of not less than 20% by volume of 100° proof malt whiskey or whiskeys distilled in pot stills at approximately 171° proof, from a fermented mash of dried malted barley, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof; and

(3) Not more than 80% by volume of neutral spirits, or whiskey distilled at more than 180° proof, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof.

BRANDIES

The following standards of identity for brandies are quoted from Regulations 5 (Article II, Sec. 21, Class 4) of the Federal Alcohol Administration.[1]

[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939.

(*a*) "Brandy" is a distillate, or a mixture of distillates, obtained solely from the fermented juice or mash of fruit (1) distilled at less than 190° proof in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to brandy; and (2) bottled at not less than 80° proof; and shall also include such distillates, aged for a period of not less than fifty years, and bottled at not less than 72° proof, in cases where the reduction in proof below 80° is due solely to losses resulting from natural causes during the period of aging.

(*b*) "Brandy," without appropriate qualifying words, or "grape brandy," is the distillate obtained from grape wine or wines under the conditions set forth in subsection (*a*) of this class, and includes mixtures solely of such distillates.

(*c*) "Apple brandy" (apple jack), "peach brandy," "cherry brandy," "apricot brandy," "orange brandy," "raisin brandy," and other fruit brandies are distillates obtained from the fermented juice or mash of the respective fresh or dried or otherwise treated fruits under the conditions set forth in subsection (*a*) of this class, and include mixtures composed wholly of one kind of such distillates. The designation shall contain the name of the fruit used, and, if other than whole fresh fruit is used, the word "dried" or such other term as may be appropriate. Brandy derived from raisins shall be designated as "raisin brandy."

(*d*) "Cognac" or "Cognac brandy" is grape brandy distilled in the Cognac Région of France, which is entitled to be designated as "Cognac" by the laws and regulations of the French Government; and includes mixtures of such brandy.

Brandies usually contain 40 to 50 per cent ethyl alcohol by volume, but may sometimes contain as little as 36 per cent alcohol by volume [paragraph (*a*) above].

A large amount of the wine manufactured in California (more than one-half, according to Goresline),[1] is distilled to produce brandy. Approximately 75 per cent of such brandy is used to fortify wines.

GINS

(*a*) "Distilled gin" is a distillate obtained by original distillation from mash, or by the redistillation of distilled spirits, over or with juniper berries and other aromatics customarily used in the production of gin, and deriving its main characteristic flavor from juniper berries and reduced at time of bottling to not less than 80° proof; and includes mixtures solely of such distillates.

(*b*) "Compound gin" is the product obtained by mixing neutral spirits with distilled gin or gin essence or other flavoring materials customarily used in the production of gin, and deriving its main characteristic flavor from juniper berries and reduced at time of bottling to not less than 80° proof; and includes mixtures of such products.

(*c*) "Dry gin," "London dry gin," "Hollands gin," "Geneva gin," "Old Tom gin," "Tom gin," and "buchu gin" are the types of gin known under such

E, H. E., Notes on Wine Manufacture in the United States, *U.S. Dept. Agr.* (mimeographed sheets), 1936.

designations, and shall be further designated as "distilled" or "compound," as the case may be.[1]

As noted above, gins contain at least 40 per cent ethyl alcohol by volume.

The basic flavoring of the best gins are produced by juniper berries, coriander seed, fennel seed, sweet orange, and cassia bark.[2]

CORDIALS AND LIQUEURS

(*a*) Cordials and liqueurs are products obtained by mixing or redistilling neutral spirits, brandy, gin, or other distilled spirits with or over fruits, flowers, plants, or pure juices therefrom, or other natural flavoring materials, or with extracts derived from infusions, percolations, or maceration of such materials, and to which sugar or dextrose or both have been added in an amount not less than 2½% by weight of the finished product. Synthetic or imitation flavoring materials shall not be included.

(*b*) "Sloe gin" is a cordial or liqueur with the main characteristic flavoring derived from sloe berries.

(*c*) Cordials and liqueurs shall not be designated as "distilled" or "compound."

(*d*) Dry cordials and dry liqueurs—The designation of a cordial or liqueur may include the word "dry" if the added sugar and dextrose are less than 10% by weight of the finished product.[3]

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[1] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Art. II, Sec. 21, Class 3, 1939.

[2] NUGEY, A. L., Modern Gin Production, *Am. Wine Liquor Jour.*, p. 42, April, 1934.

[3] U.S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Art. II, Sec. 21, Class 6, 1939.

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CHAPTER VIII

YEAST: ITS MANUFACTURE, VITAMINS, AND USES

Development of Industry.—The baking of bread has been practiced for centuries. Egyptian, Jewish, Greek, and Roman histories reveal many interesting facts concerning baking, brewing, and wine making. The Bible suggests a method for preparing leavened bread.

Mason, an Englishman, is said to have prepared the first compressed yeast in 1792.[1] No hops were used in its manufacture. Shortly thereafter, compressed yeast was known in Holland and in Germany, as well as in England. Indeed, there is some evidence that compressed yeast was made in Holland as early as 1781.

Leavened bread was prepared for many years by mixing a portion of leftover dough with the main batch and permitting the mix to stand for a while to undergo fermentation. A more modern practice was to mix residual yeast, from the brewery or distillery, with the dough. Such yeast often possessed a bitter taste due to the use of hops in malt-beverage manufacture.

The Vienna Process.—Some[2] consider that the compressed-yeast industry started with the development of the Vienna process, a modified brewing operation, about 1860. In this process, kiln-dried malt and corn were ground, mixed with water, and mashed (see chapter on Brewing). The unfiltered mash was pitched and permitted to ferment. Machines separated the portions of grain from the yeast by a process of sifting. Yields of 10 to 14 per cent on the basis of the grain were obtained along with a considerable amount (about 30 per cent) of ethyl alcohol.

Since compressed-yeast manufacturers are interested in the yield of yeast and not the alcohol, improvements in this method were made from time to time. A process for aerating mashes for yeast production was developed by Marquardt in 1879. Yields of yeast were materially increased, while those of alcohol were decreased. The filtration of the wort, the use of very dilute worts, carefully regulated aeration, and fairly low temperatures were later modifications that still further improved the process.

Many of the modern methods of compressed-yeast manufacture are protected by patents.

[1] Frey, C. N., *Ind. Eng. Chem.*, **22**: 1154 (1930).

[2] Frey, C. N., G. W. Kirby, and A. Schultz, *Ind. Eng. Chem.*, **28**: 879 (1936).

Soon after the commencement of the World War, research was carried out in Germany which led to the development of some of the modern methods of manufacture. The high cost and scarcity of grains had much to do with the instigation of this research.

Molasses-ammonia Process.—In the molasses-ammonia process, molasses (cane and sugar beet) is used as the source of carbon and mineral elements for the growth of the yeast, while the nitrogen is supplied by ammonia.

The molasses mash is carefully prepared. If the molasses is deficient in phosphorus, ammonium or calcium phosphate is added. Phosphorus is important in the synthesis of nucleoproteins by the yeast and in buffering the medium. It stimulates growth also.

In order to produce a clear solution, the molasses wort is filtered.

The pH of the clear, highly diluted mash is adjusted. The wort is then inoculated with a starter of yeast, which has been carefully prepared from a single cell of the selected yeast.

During operation, several factors must be carefully controlled. The most important of these are the aeration, the pH, the concentration of the available sugar, and the temperature.

The availability of a large supply of air stimulates the production of yeast cells. All the air used must be sterilized before passage through the mash.

The pH is maintained within a definite range, that most favorable for yeast growth (about 4.4), during the entire growth period by the addition of sulphuric acid or ammonium hydroxide. As the ammonia is used up, the mash tends to become more acid in reaction. The addition of ammonium hydroxide not only neutralizes some of the acid but also supplies more ammonium salts for the nitrogenous needs of the growing yeast.

During the process, measured portions of the molasses wort are added at specified time intervals in such quantities that the yeast produces but very little alcohol from the sugar.

It is essential to control the temperature of the mash during the growth of the yeast. This may be done by the use of cooling coils set on the inside of the covered vats, which usually have capacities for several thousand gallons. The optimum temperature for the growth of the yeast strain being used is maintained.

At the end of the growing process, yeast is separated from the wort by the use of centrifugals (or by filtration). The yeast thus obtained is washed repeatedly, until the wash water appears to be quite clear. It is then pressed.

A small amount of flour (potato, tapioca, or cereal) may or may not be added to the yeast. The flour absorbs some of the moisture from the

yeast, increases the friability, and aids in molding the yeast into small cakes. Much yeast for the baking industries is packaged without the incorporation of starch.

Irradiation.—Yeast, in aqueous suspension, is irradiated to convert ergosterol to vitamin D. Such irradiated yeast may be dried and sold for cattle fodder.

Yields.—Based on the sugar, yields of as much as 200 per cent have been secured, using the ammonia-molasses process. Reproduction of the cells is very rapid, the process requiring only a few hours for completion.

Yeast from Sulphite Liquor by the Heijkenskjöld Method.[1]—Sulphite liquor, mixed with a small quantity of molasses, serves as the source of raw material for the manufacture of yeast by the Heijkenskjöld method.

The first step in this process consists of adjusting the hot sulphite liquor to a pH of 6.0 to 6.5 with lime, powdered limestone, and sodium carbonate, while the liquor is being aerated in tanks. After the neutralization, the liquor is permitted to stand, in order that the precipitates may settle out. The clear supernatant liquor is decanted from the tanks and cooled to 28 to 30°C. by passage through coolers and is then ready for use in the manufacture of yeast.

The initial mash contains molasses mixed with sulphite liquor to yield a sugar concentration of 3 to 5 per cent. Malt sprouts may be added to this mash, if desired, to supply organic nitrogen and other nutrient substances. The mash is now inoculated with the seed yeast, a strain of *Saccharomyces cerevisiae* ("Rasse 12" of the Gärungsinstitut of Berlin is used in Finland and at the Best plant in Liverpool, Nova Scotia[2]).

Sulphite liquor is fed into the mash after the yeast has commenced to develop new cells. Ammonium sulphate and phosphates are added during the course of the process to furnish nutrient material and to maintain the desired reaction. Large quantities of filtered air are supplied, the air removing the carbon dioxide and other gases formed besides carrying out other functions. After the propagation is complete, the yeast is washed repeatedly. The final product is light in color, of high purity, and neutral in taste.[2]

The sulphite liquor used in this process contains approximately 25 kg. of sugar per 1,000 kg. of liquor, and of this amount about 16 kg. are fermentable. The sugar concentration is thus very low and would not favor the production of alcohol in large amounts.

An average yield of 160 per cent is obtained on the basis of the fermentable sugar, yields varying from 40 to 55 lb. for each ton of waste sulphite liquor.

r, E. W., *Chem. Industries*, **38**: 573 (1936).

[2] *Ibid.*

Fodder Yeast from Wood Sugar.—The dilute sugar solution prepared from wood by the Scholler-Tornesch process,[1] fortified by the addition of nitrogen, phosphorus, potassium, and magnesium in the form of salts, is used for the production of fodder yeast. Aeration, temperature, and other factors are controlled in this process as in other processes. Yields of 25 kg. or more of dry yeast may be obtained from 50 kg. of wood sugar or 100 kg. of dry wood substance.[1]

TABLE 32.—COMPOSITIONS OF SOME MEDIA FOR GROWTH OF YEAST[1]

GRAIN-WORT MEDIUM	
Corn	2 kg.
Malt	1.5 kg.
Sprouts	1.5 kg.
Concentrated hydrochloric acid	10 cc.
Tap water, to make	30 liters
MOLASSES-SALTS MEDIUM	
Beet molasses (crude)	150 g.
Calcium phosphate	1 g.
Ammonium monohydrogen phosphate	1 g.
Urea	2 g.
Concentrated sulphuric acid	2 cc.
Tap water, to make	1 liter
GLUCOSE-SALTS MEDIUM	
Commercial glucose	70 g.
Sodium chloride	2 g.
Magnesium sulphate	2 g.
Calcium chloride	0.4 g.
Potassium pyrophosphate	2 g.
Ferric pyrophosphate	0.1 g.
Ammonium chloride	0.15 g.
Copper sulphate	Trace
Urea (commercial)	2.4 g.
Tap water, to make	1 liter

[1] PAVCEK, P. L., W. H. PETERSON, and C. A. ELVEHJEM, *Ind. Eng. Chem.*, **29**: 536 (1937).

Preparation of Media.—The media of the foregoing table are prepared as follows:

1. *Grain-wort Medium.*—The finely ground corn is autoclaved at a pressure of 15 lb. per sq. in. for 0.5 hr. in the presence of about 8 liters of water. At the same time the malt and sprouts are mixed in 20 liters of water, 5 cc. of concentrated HCl is added, and the mixture is allowed to soak at 25°C. for 0.5 hr. Approximately one-half of the cooked corn is added to this malt-sprouts mixture. The temperature is then raised to 50°C. and maintained at this level for 0.5 hr. Next the rest of the corn plus an additional 5 cc. of concentrated HCl are added, and the temperature is raised to 62°C. and kept constant until the iodine test for starch is negative. The mash is filtered through burlap bags, and the

[1] SCHOLLER, H., *Chem. Ztg.*, **60**: 293 (1936).

residue is washed once with warm water. The combined filtrate is made up to 30 liters with water and autoclaved at a pressure of 15 lb. per sq. in. for 45 min. The resultant wort should contain 4.5 to 5 per cent of reducing sugar (as glucose) and have a pH of 4.4.

2. *Molasses-salts Medium.*—The molasses, calcium phosphate, and sulphuric acid are sterilized together after dissolving them in water. Sterilized urea and ammonium monohydrogen phosphate are added to the cooled solution, and the pH is adjusted to 4.4.

3. *Glucose-salts Medium.*—Solutions of glucose, sodium chloride, magnesium sulphate, calcium chloride, and copper sulphate are autoclaved at a pressure of 15 lb. per sq. in. for 1 hr. at a pH of 3.5. A sterilized solution of potassium and ferric pyrophosphates is added to the cooled main mash. If a precipitate forms it is dissolved by adding some sterilized 40 per cent sulphuric acid. A sterilized solution of ammonium chloride and urea is added finally to the main mash, and the pH is adjusted to 4.4 with sterilized 40 per cent sodium hydroxide (NaOH). Although the ratio of carbon to nitrogen in the preceding medium is 25:1, the ratio can be reduced by adding increasing quantities of ammonium chloride and urea.

Yields.—Pavcek, Peterson, and Elvehjem have reported the following yields of dry yeast grown on the media indicated:

TABLE 33.—YIELDS OF DRY YEAST[1] FROM VARIOUS MEDIA[2]

Type of yeast	Grain medium, per cent	Molasses-salts medium, per cent	Glucose-salts medium, per cent
Bakers' Yeast *A*	24.3	34.6	18.0
Bakers' Yeast *B*	42.5	33.6	34.3
Brewers' Yeast *A*	34.6	42.7	29.0
Brewers' Yeast *A* (autoclaved medium)	32.2		
Saccharomyces logos	33.1	28.0	21.4
Willia anomala	21.4	28.6	11.4
Endomyces vernalis	40.9	33.6	30.5

[1] Dry yeast is based on glucose fermented.
[2] PAVCEK, P. L., W. H. PETERSON, and C. A. ELVEHJEM, *Ind. Eng. Chem.*, **30**: 802 (1938).

Without aeration the yields were of low magnitude—approximately one-tenth of those with aeration.

Apparatus for Growing Yeast.—Figure 15 shows an apparatus suitable for growing yeast.

Some General Considerations Concerning the Production of Yeast. *Yeast.*—Selected strains of *Saccharomyces cerevisiae* are used in the manufacture of compressed yeast. The baker depends upon the yeast to

bring about definite changes in structure and flavor in the dough during a given amount of time. Failure to accomplish these changes may result in considerable loss of money. Therefore a vigorous strain that shows uniform and constant characteristics should be used for the manufacture of compressed yeast.

Nutrient Materials.—Carbon, necessary for growth and energy, is generally obtained from the sugars of the mashed grains or the molasses, although lactic acid, amino acids, and even ethyl alcohol may be used under certain conditions.

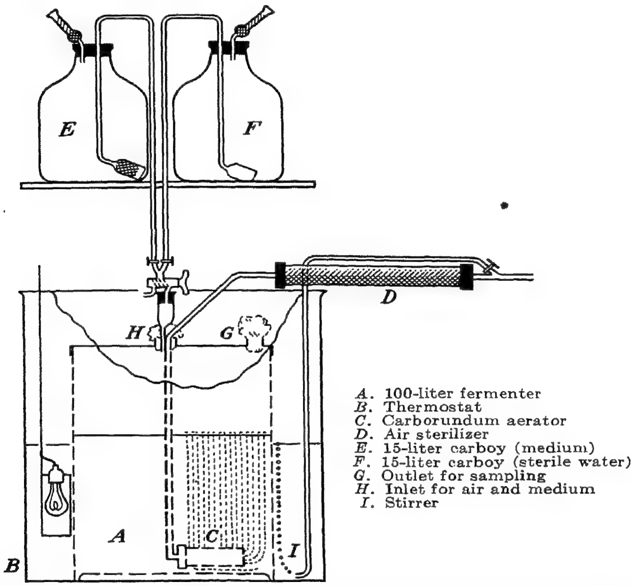


FIG. 15.—Apparatus for growing yeast. [*Courtesy of Profs. P. L. Pavcek, W. H. Peterson, and C. A. Elvehjem, Ind. Eng. Chem.* **29**: 536 (1937).]

Nitrogen is supplied commonly by the use of malt or mashed cereal grains, or by ammonium salts. Peptones, peptides, amino acids, and ammonia are absorbed by the yeast and assimilated. Nitrates and nitrites are not used by yeast, however.

Ammonium lactate, accordingly, may furnish both carbon and nitrogen.

Soybeans, freed of the bitter principles, sugar-beet juice, and various other substances have been proposed either to increase the yield of yeast or to act as the source of nutrient material.[1]

[1] CHRZASZCZ, T., and J. JANICKI, *Chem. and Ind.*, **55**: 884 (1936).

Use of Lactic Acid Bacteria.—Frequently in the manufacture of yeast from cereal grain, the filtered mash may be inoculated with a culture of *Lactobacillus delbrueckii* and maintained for a while at a temperature of 50 to 52°C. to favor the production of lactic acid. Yeasts grow well in the media containing lactic acid while contamination, especially with butyric acid organisms, is reduced. The presence of lactic acid in the yeast cake inhibits the development of bacteria.

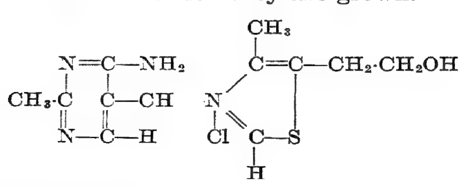
Function of Oxygen.—The exact function of oxygen in the growth of yeast is not known. Very likely its action may be due to several factors: inhibition of fermentation and increase of respiration, agitation of the medium, removal of toxic end products, and stimulation of vegetative growth.

Preserving the Yeast.—The use of a low temperature is necessary for the storage of compressed yeast. Molds and bacteria cause the cake to deteriorate rapidly at room temperature and autolysis is likely to take place.

Various methods for improving the keeping qualities of yeasts have been proposed. For example, it has been suggested[1] that washing the yeast for 1 hr. with a solution of ethyl, propyl, isopropyl, butyl, or amyl alcohol before pressing improves the keeping quality. The use of colloids—pectin, agar, gelatin, gum tragacanth, dextrin, algae extract—to remove the water from yeast, followed by drying to a definite moisture content at a temperature that will not injure the yeast, has also been proposed.

Production.—According to Frey,[2] approximately 230,000,000 lb. (115,000 tons) of yeast are produced annually in the United States, the bulk of which is used for breadmaking and for its vitamin value. Ramsbottom states that 2,200 tons of bakers' yeast was produced in Great Britain in 1930.[3]

The Vitamins of Yeast. *Vitamin B_1.*—Vitamin B_1 (thiamin, or aneurin), the antiberiberi vitamin, whose structural formula follows,[4] is produced in varying amounts by different yeasts, depending largely upon the nature of the medium in which they are grown.



[1] British Patent 406,398, 1934.

[2] FREY, *loc. cit.*

[3] RAMSBOTTOM, J., *Brit. Assoc. Advancement Sci., Annual Report,* 1936.

[4] ADDINALL, C. R., "The Story of Vitamin B_1," Merck & Co., Inc., Rahway, N.J., 1937.

According to the investigations of Pavcek, Peterson, and Elvehjem, the vitamin B_1 content of most of the yeasts grown on the same medium under similar conditions was approximately the same. The vitamin B_1 content varied widely on different media, however.

The vitamin B_1 content of the yeasts listed in the following table was approximately 10 I.U. (International Units) per g. of dry yeast for the grain medium, with the exception of *Endomyces vernalis*, which yielded approximately 7 I.U. per g. For the molasses-salts medium, the vitamin B_1 content varied from 3 to 4 I.U. (*E. vernalis*, excepted); for the glucose-salts medium, the vitamin B_1 content varied from 2.5 to 3.3 I.U. (omitting *E. vernalis*).

TABLE 34.—APPROXIMATE AMOUNT OF VITAMIN B_1 PER GRAM DRY YEAST[1]

	Grain medium, I.U.	Molasses-salts medium, I.U.	Glucose-salts medium, I.U.
Bakers' yeast *A*	<10	<3	3
Bakers' yeast *B*	10	3	<3.3
Brewers' yeast *A*	10	<3	<3.3
Brewers' yeast *A* (medium autoclaved)	10		
Saccharomyces logos	10	<3	<2.5
Willia anomala	<10	4	<3.3
Endomyces vernalis	7	5	7

[1] Data from the investigations of Pavcek and his coworkers.

The superiority of the grain medium from the point of view of vitamin B_1 production is evident. Both the grain and the molasses media contained vitamin B_1 before inoculation with yeast, while the glucose-salts medium contained none.

The indications are that yeast will abstract vitamin B_1 from the medium, when it is thus available, in preference to synthesizing it.[1] The addition of vitamin B_1 crystals, yeast concentrates of B_1, nucleic acid, or liver extract increased the yields of the vitamin produced by bakers' yeast, strain *B*, from the glucose-salts medium. (Nucleic acid, which contains no vitamin B_1, was used because it was believed that it might function as a precursor of the vitamin.[2])

Apparently yeast is able to resynthesize vitamin B_1 from its decomposition products, for the destruction of the vitamin in grain medium by prolonged heating at pH 6 did not decrease the yield of the vitamin.[2]

The vitamin B_1 potency of yeast is increased when aeration is not used, but the yield of yeast is small under such conditions.

[1] FISCHER, A. M., *Brewers Digest*, **13**: 37 (No. 10) (1938).

[2] PAVCEK, P. L., W. H. PETERSON, and C. A. ELVEHJEM: *Ind. Eng. Chem.*, **29**: 536 (1937).

Vitamin G.—Certain strains of yeasts are rich in vitamin G.

Ergosterol and Vitamin D.—Yeasts contain ergosterol, a substance which, when irradiated, forms vitamin D. The quantity of ergosterol produced by different yeasts varies. Irradiated yeast may be produced in the fresh or dry condition. Ergosterol may be extracted from the yeasts and then irradiated.

The vitamin D content of milk is increased by feeding irradiated dry yeast to cows.[1]

Bunker and Harris have reappraised vitamin D milks.[2]

Since vitamin and ergosterol production by yeast are factors subject to variation, each lot of yeast must be bio-assayed in order to determine the exact amounts of these substances present.

Yeast may be dried under carefully controlled conditions and still maintain an effective vitamin content.

The feeding of yeast to persons suffering with pellagra has brought definite relief.[3] Dried yeast is a rich source of the pellagra-preventing factor.[4]

Protein.—The protein of yeast is said to be a biologically complete protein.[5] An analysis of the dry matter of yeast indicates that protein constitutes slightly more than 50 per cent of the dry weight. As a substitute for cattle beef, yeast may be quickly manufactured from molasses and ammonia, the ammonia being synthesized by the Haber process. The proteolytic digest of yeast yields a product that is meat-like and contains vitamin G in large amounts. A yeast protein product may also be prepared by special hydrolysis. Yeast extracts are useful in culture media and for food purposes.

Nucleic acid is obtained from yeast and used for medicinal purposes.

The phosphates and potassium salts found in yeast are valuable as food components.

Fat.—See the section on fat production.

Invertase.—The enzyme invertase is produced by growing yeasts. Invertase is used by confectioners, bakers, and sirup manufacturers. It converts sucrose to glucose and fructose by inversion, thus making possible sugar content without crystallization taking place.

Medical Uses.—The use of yeast in the diet has been attended by beneficial results in many instances.

[1] Wachtel, M., *Münch. med. Wochschr.*, **76**: 1513 (1929); Steenbock, H., E. B. Hart, F. Hanning, and G. C. Humphrey, *Jour. Biol. Chem.*, **88**: 197 (1930).

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[4] *U.S. Dept. Agr., Misc. Pub.* 275, June, 1937.

[5] Frey, Kirby, and Schultz, *loc. cit.*

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CHAPTER IX

THE GLYCEROL FERMENTATION

Glycerol $\left(\begin{array}{c} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array}\right)$ is the simplest alcohol that contains three hydroxyl groups. It is a chemical substance that has many uses in industry and in the arts.

In industry, glycerol is prepared principally by the saponification of fats and oils in the making of soaps. Synthetic glycerol may be made from propylene or propane. A very small amount of free glycerol is found in palm and in some less common oils. During the World War, approximately 1,000 tons of glycerol per month were manufactured by a fermentation method, known as the "sulphite process."[1]

Historical.—In his studies of wines and beers, Pasteur found that glycerol was formed regularly by yeasts to the extent of 2.5 to 3.6 per cent on the basis of the weight of the sugar fermented.

About three years before the beginning of the World War, Neuberg and his fellow workers commenced to publish some of the results of their studies concerned with the mechanism of the ethyl alcohol fermentation by yeasts. Neuberg's scheme for the course of events during the normal fermentation is shown in Chap. IV. While experimenting on aldehyde fixation with sodium sulphite in an attempt to elucidate further facts concerning the mechanism of the fermentation, he discovered that a large increase occurred in the amount of glycerol formed when sulphite was added to the fermentation medium. He suggested the following reaction:

$$C_6H_{12}O_6 + Na_2SO_3 + H_2O \rightarrow NaHCO_3 + CH_3{\cdot}CHO{\cdot}NaHSO_3 + C_3H_8O_3$$

From the preceding equation, 100 g. of hexose theoretically yields 51 g. of glycerol when 70 g. of anhydrous sodium sulphite is used. At the same time, slightly more than 24.4 g. of acetaldehyde is fixed. The ratio of acetaldehyde to glycerol is 24.4:51, or 1:2.09.

Table 35 shows the yields of glycerol and aldehyde obtained when the proportions of sodium sulphite were varied:

E, J. W., "Glycerol and Glycols," Reinhold Publishing Corporation, New York, 1928.

TABLE 35.—EFFECT OF VARIOUS AMOUNTS OF SODIUM SULPHITE ON YIELDS OF GLYCEROL AND ALDEHYDE[1]

Na_2SO_3, parts used	Sugar, parts used	Aldehyde, parts produced	Glycerol, parts produced	Ratio of aldehyde to glycerol
33	100	11.90	23.37	1:1.96
50	100	12.52	24.86	1:1.98
75	100	13.89	27.62	1:1.98
150	100	18.65	36.90	1:1.98

[1] LAWRIE, J. W., "Glycerol and Glycols," Reinhold Publishing Corporation, New York, 1928.

An examination of this table indicates that the ratio of aldehyde to glycerol that was obtained was fairly close to the theoretical and independent of the quantity of sulphite used.

Neuberg's second and third schemes indicate the courses of the fermentations when a sulphite and an alkaline medium are used.

Methods.—Increased yields of glycerol may be secured by the use of acids; by acid salts; by neutral salts; by alkalies; or by alkaline salts.

Three well-known processes for the production of glycerol by fermentation are the sulphite process, with which the names of Connstein and Lüdecke are associated and which was used on a large scale by Germany during the World War; the Cocking and Lilly process in which a mixture of sulphite and bisulphite is used; and the alkaline process developed by Eoff in the United States.

In all these processes the basic medium contains a fermentable sugar and, if necessary, added nutrient salts. The medium is inoculated with a yeast culture and maintained at the optimum temperature for the fermentation, usually 30 to 37°C. From time to time during the fermentation, small portions of the agents that cause increased yields of glycerol are added to the mash. The normal course of the fermentation is altered.

The Sulphite Process.—The basis for this process is the fixation of acetaldehyde by sodium sulphite:

$$CH_3{\cdot}CHO + Na_2SO_3 + H_2O \rightarrow CH_3{\cdot}CHO{\cdot}HSO_3Na + NaOH$$

Normally acetaldehyde is reduced in large part to ethyl alcohol during the fermentation of sugars by yeasts. But when this hydrogen acceptor becomes fixed by sulphites, a second molecule in the mash, a triose produced from hexose, acts as the main hydrogen acceptor and becomes reduced to glycerol (see page 73 of Chap. IV for the reactions).

The effect of various concentrations of sodium sulphite on the glycerol yield is shown in the following table:

TABLE 36.—EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM SULPHITE ON YIELDS OF GLYCEROL[1]

Sodium sulphite, parts by weight	Sugar, parts by weight	Glycerol yield, based on sugar
40	100	23.1
67	100	24.8
80	100	27.3
100	100	30.1
120	100	33.0
150	100	34.6
200	100	36.7

. W., "Glycerol and Glycols," Reinhold Publishing Corporation, New York, 1928.

Increasing the amounts of sodium sulphite caused a corresponding increase in the quantity of fixed aldehyde and decreases in the yields of alcohol and carbon dioxide.

Salts giving an alkaline reaction were found to increase the yield of glycerol, but alkaline media favored the growth of contaminants. By using large amounts of sodium sulphite, the danger of infection was eliminated.

The sulphite process that the Germans used during the World War was founded on the following example:[1] Sucrose (1 kg.), nutrient salts (50 g. of ammonium nitrate and 7.5 g. of dipotassium phosphate), and sodium sulphite (400 g.) were dissolved in water (10 liters) and inoculated with fresh yeast (100 g.), the mash being permitted to incubate at 30°C. for 2 to 2.5 days.

Although beet sugar was used by the Germans in the sulphite process, it has been stated that "neither the kind of sugar nor the variety of yeast influence the fermentation."[2] Yeast may be used repeatedly, if purified between fermentations.

It is important to control the temperature of the fermentation, especially during the summer months.

Glycerol may be recovered by the following method of Connstein and Lüdecke:[2] Alcohol and acetaldehyde are separated by distillation. The sulphite in the spent slop is precipitated as calcium sulphite by the addition of calcium oxide, hydroxide, or chloride, and filtered out. Calcium salts that remain in the filtrate are treated with sodium carbonate to form the insoluble calcium carbonate, which is removed. Technically pure glycerol is obtained by distilling the liquor, which contains glycerol and sodium chloride mainly, under reduced pressure.

[1] MAY, O. E., and H. T. HERRICK, *Ind. Eng. Chem.*, **22**: 1172 (1930).

[2] LAWRIE, *op. cit.*

In order to determine the amount of glycerol present in the mash, the filtrate from which the carbonates have been removed may be reduced to a sirup by evaporation and then extracted with ethyl alcohol. Glycerol and alcohol are separated by the process of evaporation. The glycerol may then be determined by the Zeisel-Fant isopropyl iodine method or distilled and weighed as such.

For an extended discussion of the recovery and determination of glycerol, in which there are certain problems, the interested reader is referred to "Glycerol and Glycols," by Dr. J. W. Lawrie.

Theoretically, a yield of 51 parts of glycerol should be obtained from 100 parts of hexose. Yields of this magnitude are not obtained by the sulphite process, but considerable of the glycerol is lost owing to low efficiency in the recovery process. On the basis of the weight of sucrose fermented, approximately 20 to 25 per cent of glycerol, 30 per cent of alcohol, and 5 per cent of acetaldehyde were obtained by Connstein and Lüdecke. Actually it required usually 10 to 12 kg. of refined sugar to produce 1 kg. of dynamite glycerol by this process on an industrial basis.

The Cocking-Lilly Process.—This process is a modification of the sulphite process of Connstein and Lüdecke. Mixtures of normal sulphites and bisulphites of the alkali metals are added to the fermenting mash. The fermentation time is much shorter than in the normal sulphite process. Yields should be higher.

The addition of bisulphites to a fermenting mash causes the acetaldehyde to be fixed at an earlier stage in the fermentation than is usually possible and aids in neutralizing the bicarbonate formed.

Bisulphites are antiseptic in nature. Owing to their antiseptic properties, they cannot be used alone in the fermentation mash in large amounts. But they may be mixed with normal sulphites to produce a combination which is neutral to litmus and which does not demonstrate too strong inhibitory action toward the yeast.

A bisulphite is formed when sodium sulphite is added to a fermenting mash, but it reacts with acetaldehyde to form the fixation product:

$$+ H_2O + CO_2 \rightarrow NaHSO_3 + NaHCO_3$$

When a mixture of sulphites (molecule for molecule) is added to fermenting media, the reaction is as follows:

$$Na_2SO_3 + NaHSO_3 + CO_2 + H_2O \rightarrow 2NaHSO_3 + NaHCO_3$$

During the fermentation, an aqueous solution of a bisulphite (preferably sodium) is added in successive small portions, or aqueous solutions of a mixture of sulphites, containing increasing proportions of bisulphite, are added as the fermentation proceeds. The relative proportions of the sulphites should be such that the combination is neutral or approximately neutral to litmus.

The total quantity of sulphites to be added to a mash may be calculated either on the basis of the yield of glycerol desired or on the basis of the theoretical yield of glycerol.

The Eoff Process.—This process, one for producing glycerol in an alkaline medium, was developed by Eoff, Linder, and Beyer[1] of the Division of Chemistry of the Bureau of Internal Revenue. Investigations were initiated in 1917 as a result of reports that glycerol was being made by fermentation methods in Germany.

The Process in Brief.—A nutrient solution containing a sugar is inoculated with a selected "trained" yeast and incubated at 30 to 32°C. An alkaline reacting compound is added to the fermenting medium in amounts up to approximately 5 per cent, in accordance with a definite schedule. The fermentation proceeds usually from 5 to 7 days before the glycerol is recovered.

Details of the Process.—Blackstrap molasses, solutions containing mixtures of corn sugar and malt sprouts, and solutions of sucrose containing nutrient salts have been used successfully as media. The optimum concentration of sugar is 17.5 to 20 g. per 100 cc. Ammonium chloride in small amounts aids in the production of glycerol.

Two yeasts were found to produce the highest yields of glycerol: *Saccharomyces ellipsoideus* (var. Steinberg) and *S. ellipsoideus* (var. California wine yeast), the former yeast giving the best results.

Yeast that has been "trained" or acclimated to growth in an alkaline medium produces the highest yields of glycerol. The main mash is inoculated with a starter that represents approximately 10 per cent of its volume. The first culture of yeast may be grown in a small flask. Training is given to the yeast by adding 0.5 to 1 per cent of sodium carbonate (calculated on the basis of the weight of the solution) to the culture. The first effect is to stop fermentation. This is transitory. When the fermentation becomes active again, a larger volume of mash is inoculated with 5 to 10 per cent of trained yeast. Alkali is added to this mash, and when the culture becomes active a proportionally larger mash is inoculated. This process is repeated until the starter eventually secured is sufficiently large to seed the main mash. For further details, the reader is referred to the report submitted by Eoff, Linder, and Beyer to the Commissioner of Internal Revenue on May 6, 1918. The facts contained in this report were reproduced in the *Journal of Industrial and Engineering Chemistry*, **11**: 842 (1919).

Sodium carbonate in the form of soda ash is preferable for use in the fermentation on account of its comparatively low cost, although potassium carbonate produces results that are just as favorable. Sodium and

[1] Eoff, J. R., W. V. Linder, and G. F. Beyer, *Jour. Ind. Eng. Chem.*, **11**: 842 (1919).

potassium hydroxides, sodium perborate, and other substances may be used also to produce alkalinity. Not much more than 5 per cent of sodium carbonate (calculated on the weight of the mash) should be used, for otherwise the fermentation will be permanently inhibited. On the other hand, the highest possible concentration (close to 5 per cent) of the carbonate should be employed, for the yield of glycerol is increased by increasing the alkalinity of the mash almost to the limit of endurance of the yeast. The final amount of alkali in 100 cc. of mash may be equivalent to 95 cc. of normal sodium hydroxide.

As soon as the fermentation becomes vigorous, sodium carbonate is added as a solid. A precipitate forms after the addition of the carbonate, and the evolution of gas temporarily ceases. The precipitate disappears gradually, and fermentation again proceeds. Thereafter sodium carbonate is added in as large amounts and as rapidly as the yeast will tolerate. Usually the alkali is added in accordance with a definite schedule. Eoff was in favor of adding it in 5 portions: the first portion containing approximately 12.5 per cent of the total carbonate to be added; the second portion, about 22 per cent; the third, about 31 per cent; the fourth, about 22 per cent; and the fifth, about 12.5 per cent.

During the fermentation the temperature should be kept within the limits of 30 to 32°C. in order to ensure high yields.

From 20 to 25 per cent of the sugar of the mash is converted to glycerol, while considerable quantities of ethyl alcohol and carbon dioxide are produced.

Eoff and his associates obtained successful results from the fermentation of mashes of 2,000-gal. capacity.

Theoretical.—According to Neuberg's scheme for the production of glycerol in an alkaline medium, 1 molecule of acetic acid is produced with each 2 molecules of glycerol:

$$2C_6H_{12}O_6 + H_2O \rightarrow CH_3COOH + 2C_3H_8O_3 + C_2H_5OH + 2CO_2$$

The ratio of glycerol to acetic acid is 184:60, or 3.07:1. Some actual ratios varied from 2.91:1 to 3.12:1.[1]

Glycerol from Synthetic Sugar.—Lüdecke[2] has patented a process for producing glycerol from the sugar or sugar sirup derived from the condensation of formaldehyde in an alkaline solution. (For details of the condensation process, consult German Patent 590236.)

Uses of Glycerol.—Glycerol finds a wide variety of uses. These include its use as a solvent; as a sweetening agent; as a constituent of ointments, lotions, antiseptics, adhesives, and inks; as a food; and as an antifreeze agent. It is used in the preparation of biological media and

[1] LAWRIE, *op. cit.*

[2] LÜDECKE, K., German Patent 658047, Apr. 27, 1938.

nitroglycerine.[1] It may be used in the manufacture of synthetic rubber, glycerine-litharge cements, and modeling clays.[2] New uses for this trihydric alcohol are continually being found.[2]

Further Information.—Additional information concerning the glycerol fermentation may be obtained by a study of the publications listed at the end of this chapter. Much valuable data will be found in the patent literature, especially that of Germany. Some patents have not been published in the latter country owing to their secret nature.

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M. A., and J. R. MURPHY, Glycerine, *Am. Professional Pharmacist*, June, July, August, 1936.

[2] "Glycerine Facts," Glycerine Producers' Association, New York, 1938.

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CHAPTER X

FAT PRODUCTION BY YEASTS AND YEAST-LIKE MICROORGANISMS

Only under conditions of a national emergency would one ordinarily attempt to produce fat from microorganisms, for normally the demands of a country may be met by domestic production from animals and higher plants, or by importations. But when the usual supply becomes inadequate, waste carbohydrate materials may be used for the synthesis of fat by microorganisms.

Fat may be produced by a number of different microorganisms: by yeasts; by yeast-like microorganisms, such as *Oöspora* (*Oïdium*) *lactis;* and by molds (a discussion of fat production by molds appears in Chap. XXXIII).

"Fat" Defined.—The term "fat," as used in this chapter, refers to substances insoluble in water but soluble in ether and other fat solvents. In the literature, one finds that some authors use the term "lipoid" to designate the crude fat obtained from microorganisms. However, in view of the wide use of the word "fat," which is more properly restricted to mean the esters formed by combinations of fatty acids with glycerol, this term will be used throughout the present discussion to include what actually would be classified as lipids, according to Bloor.[1]

Under the term "lipid,"[1] Bloor includes the simple lipids—the fats, oils, and waxes; the compound lipids, such as the phospholipids; and the derived lipids, such as fatty acids, sterols, and alcohols.

The Fat of Yeast.—Some of the first studies concerning the nature of yeast fat were carried out by Nägeli and Loew (1878), Gérard (1895), Gérard and Darexy (1897), Hinsberg and Roos (1903, 1904) and Sedlmayer (1903). In recent years, amongst others, Smedley-MacLean and coworkers have made notable contributions to the knowledge of this subject.

In yeast fat are found palmitic, oleic, linoleic, and lauric acids; the phospholipids, lecithin and cephalin; ergosterol and zymosterol; and other components. A large proportion of the crude fat is unsaponifiable matter.

[1] BULL, H. B., "The Biochemistry of Lipids," John Wiley & Sons, Inc., New York, 1937.

Production of Fat by Yeast.—Nägeli and Loew first proved that carbohydrates could be transformed to fat by yeast.[1]

Fat production is a normal function of yeast cells, but the rate of its formation and the quantity of it stored may be increased through the use of certain optimum conditions. Factors of importance in fat production are the use of a medium rich in carbohydrate, oxygenation, and phosphates.

Oxygenation of a medium is essential if greater than normal quantities of fat are desired. Nägeli in 1878 showed that the fat content of yeasts and molds could be increased from 5 to 12 per cent when they were grown in well-oxygenated media containing an abundance of carbohydrates and a scarcity of nitrogen.

The addition of alkaline phosphates (Na_2HPO_4 and KH_2PO_4) to a well-oxygenated medium containing 4 per cent glucose caused increased fat storage in a suspension of brewery yeast.[2]

Usually the rate of fat formation was greatest at the beginning of the experiment, diminishing later. When the concentration of sugar was increased, the amount of phosphate absorbed by the yeast cells was likewise augmented.

It has been demonstrated that oxygenating a solution containing a suitable sugar increases the amount of fat stored in the suspended yeast. If yeast containing reserve carbohydrate is suspended in oxygenated water, part of the carbohydrate is converted into fat.[3]

Although investigations have been carried out using oxygenated solutions of various simple carbon compounds, thus far only ethyl alcohol and sodium acetate, two-carbon compounds, have produced significant increases in the amount of fat stored by the suspended yeast.[4] These compounds must be used in relatively dilute solution (a 0.5 to 0.6 per cent concentration of ethyl alcohol is satisfactory), because increasing the concentration of either above a low maximum leads to poor results.

The rate at which oxygen is supplied to solutions of ethyl alcohol and sodium acetate determined chiefly the amounts of lipids stored in yeast. When a solution of these compounds is not oxygenated the storage of fat and carbohydrate is not increased.

Alcohol vapor, in the presence of oxygen, leads to a deposition of lipoid material in the cells of brewery yeast and also in *Endomyces vernalis*, according to the researches of Lindner and Unger.[5] Yeast

[1] NÄGELI, C., und O. LOEW, *Ann.*, **193**: 322 (1878).

[2] SMEDLEY-MACLEAN, I., and D. HOFFERT: *Biochem. Jour.*, **18**: 1273 (1924).

[3] SMEDLEY-MACLEAN, I., *Ergeb. Enzymforsch.*, **5**: 285 (1936).

[4] SMEDLEY-MACLEAN and HOFFERT, *op. cit.*, **20**: 343 (1926).

[5] LINDNER, P., und UNGER, *Wochschr. Brau.*, **36**: 188 (1919); LINDNER, P., *Zeit. angew. Chem.*, **35**: 110 (1922).

(Frohberg type), when grown in agar in a chamber containing the vapors of ethyl alcohol and oxygen, showed definite increases in fat content.[1] By keeping the moisture content of the yeast low through the use of a drying atmosphere, the highest yields of fat were obtained in the presence of alcohol vapor. Substitution of ethyl alcohol with propyl alcohol in control experiments yielded negative results.

The following table indicates the average increases in the fat content of samples of yeast incubated in oxygenated water and in oxygenated solutions of N/14 acetates.[2]

TABLE 37.—AVERAGE INCREASES IN FAT CONTENT OF YEAST IN VARIOUS SOLUTIONS

Solution	Percentage Increase in Fat Content
Water	41
Potassium acetate	180
Sodium acetate	160
Magnesium acetate	118
Calcium acetate	100

When phosphate is added to a 0.6 per cent solution of sodium acetate, there is no increase in the quantity of fat produced from the acetate.

Fat is produced from sodium lactate and sodium pyruvate in oxygenated solutions containing yeast, according to Smedley-MacLean and coworkers. Shaking the solutions of these compounds increased the quantity of fat and carbohydrate stored. Lactic acid, or lactate, usually yields carbon dioxide and small amounts of volatile acids in addition to the stored products. Pyruvate is not so effective as lactate in forming storage compounds.

No increases in the lipid content of yeast, not equally well obtained by oxygenating a suspension of yeast in water, were observed by oxygenating solutions of the sodium salts of citric, crotonic, fumaric, gluconic, levulic, maleic, or succinic acids; acetoin; 2:3-butyleneglycol; or methylethyl ketone.[3]

The addition of calcium or magnesium ions to an oxygenated solution of glucose significantly decreased the quantity of lipids usually stored in yeast. Likewise, the addition of these ions to oxygenated solutions of acetates caused a decrease in the amount of lipid material ordinarily formed from the acetates.[3]

Fat Production by Endomyces vernalis.—Considerable research was undertaken by Lindner and his associates at the Institut für Gärungsge-

[1] HALDEN, H., *Biochem. Zeit.*, **225**: 249 (1934).
[2] MACLEOD, L. D., and I. SMEDLEY-MACLEAN, *Biochem. Jour.*, **32**: 1571 (1938).
[3] *Ibid.*

werbe of Berlin at the time of the World War in an effort to produce fat from *Endomyces vernalis* on a successful economic basis.[1]

Endomyces vernalis grows as a mat or skin over the surface of a liquid medium. For fat production, oxygen is essential. However, agitation of the medium is not beneficial.

Several carbohydrates are assimilated but not fermented. Since fat storage is sought, this is a desirable feature of the organism. Molasses, cellulose waste, hydrolyzed wood, and other media containing an assimilable source of carbohydrate may be used as raw materials for fat production.

Waste sulphite liquor, fortified with nitrogenous substances and the necessary salts, is a favorable raw material. Nitrogen-containing substances may include yeast water, ammonium salts, urea, urine, molasses slop, extracts of grains, or other products. Potassium chloride, primary potassium phosphate, and magnesium sulphate may be used as salts.

An abundance of a suitable carbohydrate is essential for the maximum production of fat.

The optimum temperature for growth is 15 to 20°C., although a temperature as low as 10°C. may be used.

In the production of fat by *E. vernalis*, two phases may be recognized in the incubation period: the phase during which the principal growth of the organism occurs (2 to 3 days under optimum conditions) and the phase of principal fat formation, which generally requires 6 to 8 days in addition. The phase of growth has been designated as the "protein generation" by Lindner. During this phase, the organism may be satisfactorily used for inoculation purposes. For growth the organism may be cultivated in a medium rich in nitrogen but poor in carbohydrates, but for fat production ("fat generation") a medium rich in a carbohydrate is essential. Thus is illustrated the difference between the optimum conditions for growth and optimum conditions for the production of a desirable end product.

Though many efforts were made to find satisfactory methods for producing fat from *E. vernalis*, only two methods were carried out on a large scale: the floor process and the pan process.

1. *The Floor Process.*—In this process, inert materials such as chopped straw or coarse sawdust were washed, impregnated with the nutrient medium, and sterilized. The impregnated inert material was spread in thin loose heaps over the floor and inoculated with a fine suspension of *E. vernalis*. The heaps were turned several times a day and occasionally sprayed with water. At 11°C., fat production was completed in approximately 12 days; at 20°C., in 8 to 10 days. The fungus was dried at a temperature of not above 50°C.

[1] FINK, H., H. HAEHN, und W. HOERBURGER, *Chem. Ztg.*, **61**: 689, 723, 744 (1937).

The following data (Lindner) are taken from a test in which the inert material was impregnated with a 25 per cent molasses solution and inoculated with *E. vernalis:*

TABLE 38.—DATA ON FAT PRODUCTION BY *Endomyces vernalis*

Amount of sugar used	12.5 kg.
Sugar used in CO_2 production	4.16 kg.
Sugar left for fat formation	8.34 kg.
Theoretical yield of fat	3.33 kg.
Actual yield of fat	0.95 kg.
Percentage of theoretical yield	28.5
Yield on basis of sugar used, per cent	7.6

The amount of sugar converted to fat by this process was small. Furthermore, difficulties were encountered in separating the fungus from the inert material, frequent attention was necessary during growth of the organism, and infection, especially with *Torula*, was prevalent. There was, however, adequate surface exposure.

2. *The Pan Process.*—In this process, which was carried out on a large scale, the organism was grown in large flat pans containing a shallow layer (1 to 2 cm. in depth) of a sterilized nutrient solution of sugar.

In order to conserve space and to facilitate handling, the pans were placed one above the other in frames. No covers were placed over the pans.

When the mat, or growth, on the surface of the solution in the pan became well-developed, a large part of the culture solution was carefully run off and replaced with fresh nutrient sugar solution. After the maximum fat formation (usually in 7 to 8 days), the spent culture solution was drawn off and the mat carefully washed by repeated underlayerings with water. The mats thus obtained, rich in fat, were used as a paste. Such paste was designated, for example, as "Evernal" or "Myceta." The protein content of such pastes was valuable.

In this process sugar utilization was nearly complete, but the necessary use of much hand labor increased the costs, while infection by yeasts, molds, or bacteria was a very serious matter in some cases.

Recovery of Fat.—Fat may be obtained from *E. vernalis* by one of several methods: by chemical means, by extraction with ether, and by an autolytic process. In the chemical method the fungus mat is treated with warm dilute hydrochloric acid to decompose the cells. The fat, obtained as neutral fat, may be used for food. The efficiency of the process is high.

The cells are ground with sand to disintegrate them before extraction with ether. Fat so obtained is used industrially. The efficiency of this process is not high.

Self-digestion is permitted at approximately 50°C. for 2 to 3 days in the autolytic process. The fat is recovered from the autolyzate.

Preservation of the Fat.—The fat keeps well if oxygen is completely excluded.

Fat Production by Oöspora (Oïdium).—In the year 1926, Chapman isolated a species of *Oöspora* (*Oïdium*) from a sewer blocked by the growth of this organism. Culture of this species in a nutrient solution led to the formation of a thick film within 2 days, which contained 10 per cent fat and 50 per cent protein. The flavor and odor of the film resembled cream cheese.[1]

Out of 50 strains of *Oöspora* (*Oïdium*) *lactis* examined—10 of which produced considerable quantities of fat—Fink, Haeseler, and Schmidt[2] selected two, strains *A* and *B*, which reproduced well and produced good yields of fat. Of these two strains, strain *A* was preferred, for it was less sensitive to higher temperatures and it reproduced more uniformly.

Oöspora lactis, or, as it has been commonly designated in the past, *Oïdium lactis*, is frequently found in Camembert cheese[3] and in some butters and may be a cause of spoilage of cream cheeses. It is quite resistant to heat and infection by other microorganisms. This attribute of growing in a medium that has become infected by other microorganisms and, at the same time, giving good results makes the mold especially desirable.

Optimum Conditions for Fat Production.—Since *Oöspora lactis* occurs naturally in dairy products, whey is an excellent source of raw material. Whey may be used alone or enriched with other nutrient substances. Urea, ammonium sulphate, or ammonium acetate are good sources of nitrogen, while primary potassium phosphate (KH_2PO_4) and magnesium sulphate are excellent sources of potassium, phosphate, magnesium, and sulphur.

As an illustration of the nature of the nutrient medium and the results obtained when *Oöspora lactis* is grown in Jena flasks, the following experiment is quoted:[4] The nutrient solution contained 2 liters of whey, 2 g. of ammonium sulphate, 1 g. of primary potassium phosphate, and 0.5 g. of magnesium sulphate. In each of a series of Jena flasks, 250-cc. portions were placed. Data for the experiment are shown in Table 39.

An examination of this table shows that under the conditions of the experiment the maximum yield of crude fat was obtained in 6 days.

[1] Ramsbottom, J., *Brit. Assoc. Advancement Sci.* (*Annual Report*), Sept. 10, 1936.

[2] Fink, H., G. Haeseler, und M. Schmidt, *Zeit. Spiritusind.*, **60**: 74, 76–77, 81–82 (1937); Fink, H., H. Haehn, und W. Hoerburger, *Chem. Ztg.*, **61**: 744–747 (1937).

[3] Hammer, B. W., "Dairy Bacteriology," 2d ed., John Wiley & Sons, Inc., New York, 1938.

[4] Fink, Haehn, und Hoerburger, *loc. cit.*

Other experiments by Fink, Haeseler, and Schmidt have indicated that the shallow pan or dish was superior to the Jena flask as a container. The optimum temperature for fat production was 25 to 30°C.

A sugar concentration of 4 to 6 per cent was satisfactory for both strains *A* and *B*.

Using strain *A*, Fink and his coworkers obtained yields of 12.5 to 14.34 g. of crude fat within 5 days from 100 g. of sugar in whey enriched ith ammonium sulphate, potassium chloride, and magnesium sulphate.

TABLE 39.—DATA ON FAT PRODUCTION BY *Oöspora lactis*[1]

Age, days	Yield in dry material, grams	Nitrogen, per cent of dry substance	Crude protein extraction, per cent of dry substance	Crude fat, per cent of dry substance	Total yield of crude fat, grams	pH of the nutrient solution
2	1.777	5.40	33.75	7.5	0.133	5.0
3	3.238	3.32	20.75	10.8	0.349	(6.5)
4	3.968	3.15	19.69	13.4	0.531	5.0
5	4.771	2.98	18.62	16.7	0.796	5.0
6	5.729	2.81	17.56	22.5	1.290	7.1
7	6.147	2.76	17.25	22.0	1.352	7.7
12	5.886	2.85	17.81	19.6	1.152	8.3
16	5.710	3.15	16.69	16.6	0.948	8.1

H. HAEHN, und W. HOERBURGER, *Chem. Ztg.*, **61**: 744–747 (1937).

These yields, according to Fink and his associates, were better than those obtained by Geffers[1] from pure wheys, calculated on the sugar utilized, when using strains of *Oöspora wallroth*, strains similar to *Oöspora lactis*, and were secured in a shorter period of time. However, Geffers obtained yields as high as 50 per cent of the dry weight of *Oöspora wallroth*, when using lactose.

For a further study of fat production by microorganisms, the reader is referred to the references immediately following and to Chap. XXXIII.

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[1] GEFFERS, H., *Arch. Mikrobiol.*, **8**: 66–98 (1937).

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CHAPTER XI

THE BACTERIA

Bacteria, like molds, because of their great range of types and the variety of their action, may be very useful, extremely harmful, or, so far as present knowledge goes, of no particular significance to man. It is well known that bacteria cause diseases in animals and in plants, decomposition of foodstuffs, and are responsible for many biochemical changes in nature. Some of the useful applications of bacteria are considered in the chapters that follow.

The bacteria that are used in industry may be divided by their relation to free oxygen into two main groups: the anaerobic bacteria and the aerobic bacteria. The former group, which may be considered to include also the microaerophiles, includes the largest number of useful types of bacteria from the industrial standpoint. Bacteria active in producing butanol, acetone, ethanol, isopropanol, lactic acid, fermented milk products, cheese, and other substances of recognized or potential value are included in this group, although not all the organisms concerned in the production of some of these compounds are anaerobic in character. The outstanding aerobic bacteria include the well-known *Acetobacter* group of bacteria, some of which have the ability to produce acetic acid, gluconic acid, dihydroxyacetone, sorbose, and other substances from suitable substrates by oxidative means. Among aerobes, also, may be placed the organisms concerned in the acetone-ethanol fermentation.

Table 40 gives data concerning some important fermentation bacteria, the fermentations with which they are associated, their oxygen relationships, and the optimum temperatures for their growth and biochemical activity.

Table 41 lists some of the products of bacterial fermentation, some of the materials from which these are formed, and the general class of the bacteria concerned.

TABLE 40.—IMPORTANT FERMENTATION BACTERIA: FERMENTATIONS, RELATION TO OXYGEN, AND OPTIMUM TEMPERATURES FOR GROWTH

Family	Genus and species	Fermentations with which commonly associated	Relationship to air[1]	Optimum temperature, °C.[1]
Acetobacteriaceae Bergey, Breed, and Murray	*Acetobacter hoshigaki*	Gluconic acid	Aerobic	30–35
	A. suboxydans	Dihydroxyacetone, sorbose, acetic acid, gluconic acid, etc.	Aerobic	30
	A. xylinum	Sorbose, acetic acid, etc.	Aerobic	28
	Bacterium curvum	Vinegar (quick process)	Aerobic	25–30
	Bact. orleanense	Vinegar (Orleans and quick processes)	Aerobic	20–30
	Bact. schutzenbachii	Vinegar (quick process)	Aerobic	25–27.5
Lactobacteriaceae Orla-Jensen:				
Tribe *Streptococceae*	*Leuconostoc mesenteroides*	Sauerkraut	Facultative aerobic	21–25
	Streptococcus lactis	Lactic acid	Facultative aerobic	
Tribe *Lactobacilleae*	*Lactobacillus acidophilus*	Acidophilus milk	Microaerophilic	37
	Lactobacillus bulgaricus	Milk products, lactic acid	Aerobic to anaerobic	45–50
	L. brevis	Milk products, sauerkraut, ensilage, etc.		30
	L. casei	Lactic acid	Microaerophilic	30
	L. delbrueckii	Lactic acid	Microaerophilic	45
	L. leichmannii	Lactic acid	Microaerophilic	36
	L. plantarum	Sauerkraut, pickles, milk products, etc.	Microaerophilic	30
	Propionibacterium freudenreichii	Swiss cheese, propionic acid	Anaerobic	
	P. shermanii etc.	Propionic acid, etc.	Anaerobic	
Bacillaceae Fischer:				
Genus I	*Bacillus macerans* Schardinger	Ethanol-acetone	Aerobic	42–45
	B. acetoethylicus Northrop et al. (probably identical with *B. macerans*)	Ethanol-acetone	Aerobic	40–43
Genus II	*Clostridium acetobutylicum* McCoy, Fred, Peterson, & Hastings	Butanol-acetone	Anaerobic	37
	Cl. butyricum Prazmowski	Butanol-acetone	Anaerobic	30–37
	Cl. felsineum	Retting, butanol-acetone	Anaerobic	37

[1] Data secured from "Bergey's Manual of Determinative Bacteriology," 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

TABLE 41.—PRODUCTS OF BACTERIAL FERMENTATION

Fermentation product	Materials from which formed	Organisms causing change
Acetaldehyde	Sugars, ethanol, pyruvic acid, etc.	Acetic acid and butanol-acetone bacteria, *Escherichia coli*, etc.
Acetic acid	Ethanol, glucose, cellulose, etc.	Acetic acid, heterofermentative lactic acid, butanol-acetone, isopropanol, propionic acid, and cellulose-fermenting bacteria, *E. coli*
Acetoacetic acid	Acetic acid	Butanol-acetone bacteria
Acetone	Starch, sugars, acetoacetic acid, acetic acid, isopropanol	Butanol-acetone, ethanol-acetone, isopropanol, and acetic acid bacteria
Acetylmethylcarbinol	2:3-butyleneglycol, sugars, pyruvic acid, etc.	*Aerobacter aerogenes*, butanol-acetone and acetic acid bacteria
Butanol	Starch, sugars, butyric acid, etc.	Butanol-acetone and isopropanol bacteria
Butyric acid	Starch, sugars, cellulose, acetic acid, etc.	Butyric acid and cellulose-fermenting bacteria
2:3-butyleneglycol	Glucose, diacetyl	*A. aerogenes*
Carbon dioxide	Starch, sugars, etc.	Butanol-acetone, *A. aerogenes*, *E. coli*, and many other bacteria
Dihydroxyacetone	Glycerol	Acetic acid bacteria
Ethanol	Sugars, starch, cellulose, acetaldehyde, etc.	*Termobacterium mobile* Lindner, butanol-acetone, ethanol-acetone, heterofermentative lactic acid and cellulose-fermenting bacteria; *E. coli*, etc.
Formic acid	Glucose, cellulose, etc.	Butyric, cellulose-fermenting bacteria, *E. coli*, etc.
Fructose	Mannitol	Acetic acid bacteria
Galactonic acid	Galactose	Acetic acid bacteria
Galactose	Dulcitol	Acetic acid bacteria
Gluconic acid	Glucose	Acetic acid bacteria
Glycerol	Glucose, etc.	Heterofermentative lactic acid bacteria
Glycerophosphoric acid	Triosephosphoric acid	Lactic acid bacteria, *E. coli*
Hexosediphosphate	Glucose	Lactic acid bacteria, *E. coli*
Hydrogen gas	Glucose, lactose, etc.	Butyric acid bacteria, colon-aerogenes group, and other bacteria
Isopropanol	Starch, sugars	Isopropanol-acetone bacteria
2-Ketogluconic acid	Gluconic acid (glucose)	Acetic acid bacteria
5-Ketogluconic acid	Gluconic acid (glucose)	Acetic acid bacteria
Kojic acid	Glucose, fructose, etc.	Acetic acid bacteria
Lactic acid	Starch, sucrose, glucose, fructose, etc.	Lactic acid, propionic acid, and cellulose-fermenting bacteria; *E. coli*, etc.
Mannitol	Fructose	Heterofermentative lactic acid bacteria
Mannonic acid	Mannose	Acetic acid bacteria
Mannose	Mannitol	Acetic acid bacteria
Methylglyoxal	Sugars, hexosediphosphate, etc.	Acetic acid, butanol-acetone, lactic acid and propionic acid bacteria; *E. coli*
Phosphoglyceric acid	Sugars, hexosediphosphate	Lactic acid bacteria, *E. coli*
Propanol	Propionic acid	Butanol-acetone bacteria
Propionic acid	Glucose, propanol, lactic acid, glycerol, pyruvic acid	Propionic acid and acetic acid bacteria
Propionaldehyde	Glucose, glycerol	Propionic acid bacteria
Propylene glycol	Rhamnose	*Bact. rhamnosifermentans*
Pyruvic acid	Sugars	Butyric, lactic, and propionic acid bacteria; *E. coli*
Sorbose	Sorbitol	Acetic acid bacteria
Succinic acid	Glucose, fructose, lactose, and other sugars	Propionic acid bacteria, *E. coli*, etc.

CHAPTER XII

THE ACETONE-BUTANOL FERMENTATION

There are a number of closely associated fermentations brought about by bacteria, which differ in respect to the quantity and nature of the end products and the conditions necessary for their successful termination. The most important of these fermentations is that in which the main end products are butanol, acetone, and ethanol (neutral solvents); acetic and butyric acids; and carbon dioxide and hydrogen gases. A second important fermentation gives rise to acetone and ethanol as the chief end products of value. In a third type of fermentation, butanol, isopropyl alcohol, and acetone are produced.

Historical.—Normal butyl alcohol was discovered as a regularly occurring constituent of fusel oil by Wurtz in 1852. Pasteur, however, was the first investigator to show that butyl alcohol was a direct product of fermentation. His findings were based on the results of the butyric fermentation of lactic acid and calcium lactate. In a report announcing his discovery he said:

"M. Pasteur . . . croit pouvoir affirmer que l'alcool butylique est un produit ordinaire de la fermentation butyrique."

Fitz published a series of articles on fermentations from 1876 to 1884.[1] Among these, he discussed *Bacillus butylicus*. This organism was a sporeformer and produced butyl alcohol, butyric acid, and small amounts of ethyl alcohol, chiefly, with the two gases, carbon dioxide and hydrogen. Glycerin, mannite (mannitol), and sucrose were fermented. Sucrose was inverted by an enzyme secreted by the bacillus. Since the organism did not form suitable enzymes for hydrolyzing starch and lactose, these materials were not fermented in their native conditions.

In 1887, Gruber[2] described three strains of organisms under the name of *B. amylobacter* (*Clostridium butyricum*). Each of these strains formed butyric acid and butyl alcohol from carbohydrates.

Botkin[3] isolated an anaerobic spore-forming bacillus, which he described in 1892. This organism produced butyl and ethyl alcohols and did not ferment cellulose. According to Botkin, this organism is

[1] Reilly, J., J. Hickinbottom, F. R. Henley, and A. C. Thaysen, *Biochem. Jour.*, **14**: 229 (1920); E. McCoy, E. B. Fred, W. H. Peterson, and E. G. Hastings, *Jour. Infectious Diseases*, **39**: 457 (1926).

[2] Gruber, M., *Centr. Bakt. Parasitenk.*, **1**: 367 (1887).

[3] Botkin, S., *Zeit. Hyg. Infectionskrankh.*, **11**: 421 (1892).

quite widely distributed in nature and may be isolated from garden soil, milk, and other sources.

B. orthobutylicus, an anaerobe isolated from a calcium tartrate fermentation, was described by Grimbert[1] in 1893. This organism fermented glycerin, mannite, glucose, invert sugar, sucrose, lactose, maltose, galactose, arabinose, starch, potatoes, dextrin, and inulin, but did not ferment calcium lactate, calcium tartrate, or trehalose. Butanol; a little isobutyl alcohol; butyric, acetic, and, in some cases, formic acids; and carbon dioxide and hydrogen gases were produced during the fermentation. Grimbert differentiated his organism from *B. butylicus* of Pasteur, *B. amylobacter* of Van Tieghem, *B. butylicus* of Fitz, and *Bacille amylozyme* of Perdrix. He studied the effect of age, the condition of cultivation, and the duration of the fermentation on the proportion of fermentation products and observed with care the reaction, the concentration of carbohydrate compounds, and the use of calcium carbonate in the control of acidity.

In a report on butyl alcohol bacteria, Beijerinck[2] described species named by him *Granulobacter butylicum*, etc. (*Granulobacter* is a term applied to microorganisms that demonstrate a blue color with iodine and show a distinct "swelling of the cell at sporulation.") *Granulobacter* replaced the term *Clostridium*.

Duclaux[3] in his "Sur la nutrition intracellulaire," discussed *Amylobacter butylicus*, an organism isolated from potato. The principal products of the fermentation were butyl alcohol, acetic and butyric acids (and lactic acid, sometimes), and the gases, carbon dioxide and hydrogen, in general. He found that the use of calcium carbonate in media caused the production of acids, while its absence favored the production of alcohols. In the same paper, he discussed also *A. ethylicus*, an organism likewise isolated from potato, which produced ethyl alcohol, acetic and lactic acids, and carbon dioxide and hydrogen gases.

In 1897, Emmerling[4] described a facultative anaerobe that produced butyl alcohol from various carbohydrate substances. The fermentation of 100 g. of glycerin yielded 6.3 g. of pure butyl alcohol; that of 100 g. of mannite, 10.5 g. of butyl alcohol. Butyric acid was obtained in all fermentations, and ethyl alcohol when glucose was fermented.

Grassberger and Schattenfroh[5] in 1902 reported the isolation of a motile butyric acid organism from the soil; they claimed that this organ-

[1] Grimbert, M. L., *Ann. Inst. Pasteur*, **7**: 353 (1893).

[2] Beijerinck, M. W., *Verhandel. Akad. Wetenschappen Amsterdam Afdeel. Natuurkunde*, 2e Sectie, **1**, no. 10 (1893).

[3] Duclaux, E., *Ann. Inst. Pasteur*, **9**: 811 (1895).

[4] Emmerling, O., *Ber.*, **30**: 451 (1897).

[5] Grassberger, R., und A. Schattenfroh, *Arch. Hyg.*, **42**: 219 (1902).

ism produced butyl alcohol. No alcohol was secured in some later experiments, however.

Winogradsky[1] reported in 1902 on *Cl. pastorianum,* describing its morphology and its properties as a producer of butyric acid. Butyl alcohol, ethyl alcohol, and acetic and butyric acids were produced from the carbohydrates, sucrose and dextrose.

Acetone was first discovered as a fermentation product by Schardinger in 1905. Schardinger[2] described the morphology and biology of the organism, which was named *B. macerans.* Acetone, ethyl alcohol, and acetic and formic acids were produced in fermentation by this organism. Potatoes, or a potato starch medium, with peptone and calcium carbonate were the best substrates found for producing acetone. Using a temperature of 37°C. and permitting the fermentation to continue for 6 days, Schardinger obtained 6.9 per cent by weight of acetone and 20.89 per cent by weight of ethyl alcohol from potato starch.

Buchner and Meisenheimer,[3] using the *B. butylicus* of Fitz, secured yields of 19.6 and 10.4 g. of *n*-butyl and ethyl alcohols, respectively, from 100 g. of glycerin. From glucose, low yields of these solvents were obtained per 100 g. (0.7 and 2.8 g.), but there were large quantities of butyric and acetic acids formed (26.0 and 7.5 g., respectively). Calcium carbonate was used in the medium in both cases.

First Successful Commercial Processes.[4]—The need for a synthetic rubber supplied the impetus that resulted in the first successful commercial process. Rubber was synthesized in 1860, but research was continued along this line for many years because it was believed that the natural supply of rubber would not be sufficient to meet the demand.

The year 1909 was a very active one in synthetic rubber reasearch, especially in England, Germany, and Russia, but after this year the production of plantation rubber was such that attempts to produce rubber synthetically no longer seemed feasible.

During the period of greatest activity, the firm of Strange and Graham, Ltd., carried out the most of the investigation in England. Prof. Perkin and his assistant, Weizmann, of Manchester University, and Prof. Fernbach with his assistant, Schoen, of the Pasteur Institute, were employed by the firm to carry on the research.

Synthetic rubber at this time was obtained through the polymerization of isoprene or butadiene. These compounds were best prepared

[1] Winogradsky, S., *Centr. Bakt. Parasitenk.*, Abt. II, **9**: 43, 107 (1902).

[2] Schardinger, F., *Centr. Bakt. Parasitenk.*, Abt. II, **14**: 772 (1905).

[3] Buchner, E., und J. Meisenheimer: *Ber.*, **41**: 1410 (1908).

[4] Gabriel, C. L., *Ind. Eng. Chem.*, **20**: 1063 (1928); Kelly, F. C., "One Thing Leads to Another," Houghton Mifflin Company, Boston, 1936; Arzberger, C. F., W. H. Peterson, and E. B. Fred, *Jour. Biol. Chem.*, **44**: 465 (1920).

from isoamyl alcohol and *n*-butyl alcohol, respectively. Isoamyl alcohol was secured from fusel oil, produced in the ethyl alcohol fermentation, of which it constituted approximately 87 per cent. Butadiene was eventually considered the best material to use, and the Germans made much rubber from it synthetically during the World War.

Fernbach and Weizmann in 1911 discovered bacteria that fermented potato starch, yielding amyl alcohol as one of the end products. Butyl alcohol, ethyl alcohol, and acetone were later found in the same fermentation. Fernbach classed the organism responsible for the fermentation as a "bacillus of the type Fitz."

Weizmann in 1912 left the firm of Strange and Graham, Ltd., and continued independent research on fermentation. He isolated an organism which produced nearly four times as much acetone as the Fernbach organism and which was able to ferment starches other than potato starch. To Weizmann's organism, the name *B. granulobacter pectinovorum* was given, but this was later changed to *Cl. acetobutylicum*, Weizmann.

During the years 1913 and 1914, the firm of Strange and Graham, Ltd., established plants at Rainham and King's Lynn for producing solvents by fermentation.

With the advent of the World War, it became necessary to seek a new means of producing acetone for use in the manufacture of cordite, an explosive, and "dopes" for airplane wings. The government made a contract with Strange and Graham, Ltd., to supply the acetone, but the demands could not be met with the use of potatoes as a raw material. At this time, Weizmann's bacillus came to the attention of the government and an order was issued to replace the organism then being used by his organism. Maize was used as a raw material. Distilleries were taken over in England and Canada, and one was rather unsuccessfully started in India.

After war was declared with Germany, the United States purchased two distilleries at Terre Haute, Ind., and established there the Weizmann process. With the termination of the World War, the plants were closed because acetone was no longer needed in large quantities and butanol never had been much in demand.

It was not long, however, before a demand sprang up for *n*-butyl alcohol in the manufacture of automobile lacquers, for *n*-butyl acetate was found to be superior to amyl acetate. An American corporation was organized to carry on the acetone-butanol fermentation in this country. Basic patent rights to the Weizmann and Ricard processes of a world-wide nature were secured, and a new plant was constructed and operated at Peoria, Ill.

In 1918, Higgins[1] reported the construction by the Hercules Powder Company of a plant for fermentation of kelp on the coast of Southern California. The seaweed was gathered into ships, macerated, pumped from the ships to a tank at the factory, diluted with water, and fermented at 90°F. with addition of limestone. Acetate of lime was the chief product, but butyric acid was also produced. Acetone was manufactured from the acetate of lime.

During the year 1919 several papers concerning the acetone and acetone-butanol fermentations were published. Nathan[2] described the

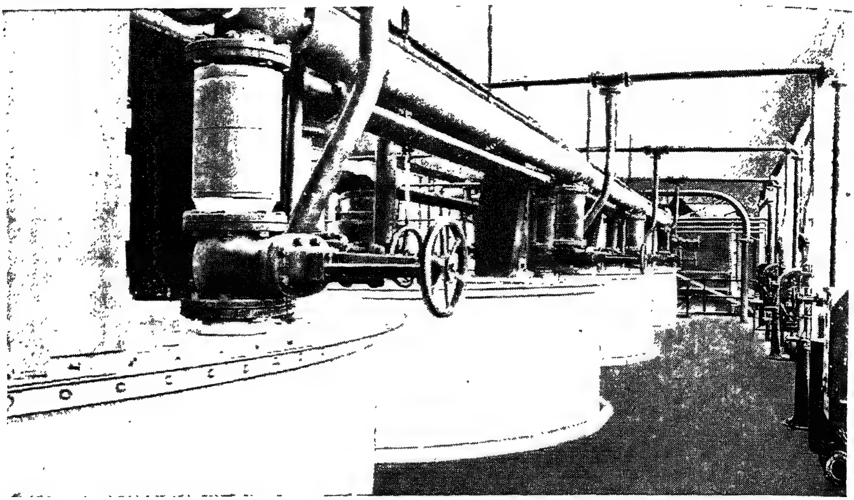


FIG. 16.—Mash tuns for mixing degermed corn meal and water. (*Courtesy G. O. Lines, Commercial Solvents Corp.*)

manufacture of acetone by the Weizmann process from such raw materials as corn and horse chestnuts. Gill[3] described in detail the acetone fermentation by the Weizmann organism and its technical application. In another report he discussed the production of acetone and *n*-butyl alcohol from horse chestnuts by fermentation.[4] Features of the fermentation of horse chestnuts, such as the long period of fermentation, frothing during fermentation and distillation, and the unhealthy appearance of the bacteria were ascribed to the presence of aesculic acid or aesculin. By removing as much of the husk as possible, the fermentation was

[1] HIGGINS, G. A., *Ind. Eng. Chem.*, **10**: 858 (1918).
[2] NATHAN, F., *Jour. Soc. Chem. Ind.*, **38**: 271–273T (1919).
[3] GILL, A., *Jour. Soc. Chem. Ind.*, **38**: 273–282T (1919).
[4] GILL, A., *Jour. Soc. Chem. Ind.*, **38**: 411–412T (1919).

improved. Yields from horse chestnuts averaged about 18 per cent on the basis of dry meal in comparison to 24 per cent from maize.

Industrial Process.—In the industrial manufacture of acetone and butanol by fermentation it is usually necessary to give the raw material some kind of preliminary treatment. When corn is used as the source of carbohydrate, the germ is removed, and the kernels are ground to a coarse meal, for, although the germ is of no value in the ensuing fermentation, it contains considerable oil of commercial importance. The ground meal is mixed with water to give a concentration of 6 to 8 per cent (see

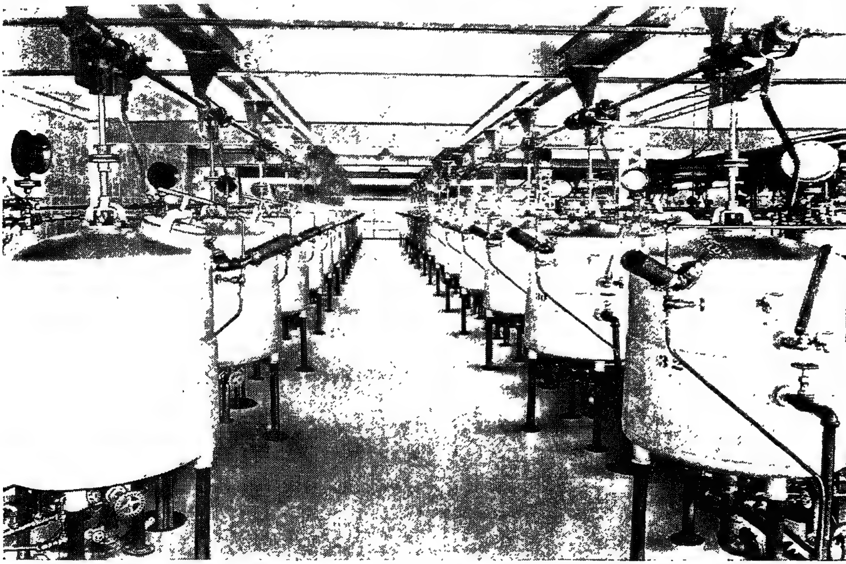


FIG. 17.—Propagation of bacteria. Eighty-gallon culture tanks. (*Courtesy G. O. Lines, Commercial Solvents Corp.*)

Fig. 16). It is then cooked, with agitation, using a steam pressure of 30 lb. for 2 hr. The starch is rendered soluble and the mash sterilized during this treatment. The cooked mash is blown aseptically through coolers, which reduce the temperature of the mash to about 37°C., to covered fermenters of large capacity where it is inoculated with starters (see Fig. 17) and allowed to ferment for 48 to 72 hr. Figure 18 illustrates the general procedure followed in an industrial process.

It is essential to sterilize all the fermenters, pipes, pipe connections, and equipment with which the mash or butanol organisms may come into contact, for contamination in this fermentation is usually a very serious matter that may involve considerable losses.

Details of the Process. *Microorganisms.—Clostridium acetobutylicum* McCoy, Fred, Peterson, and Hastings; *Cl. butyricum* Prazmowski;[1] and other bacteria may be used for the production of neutral solvents by fermentation.

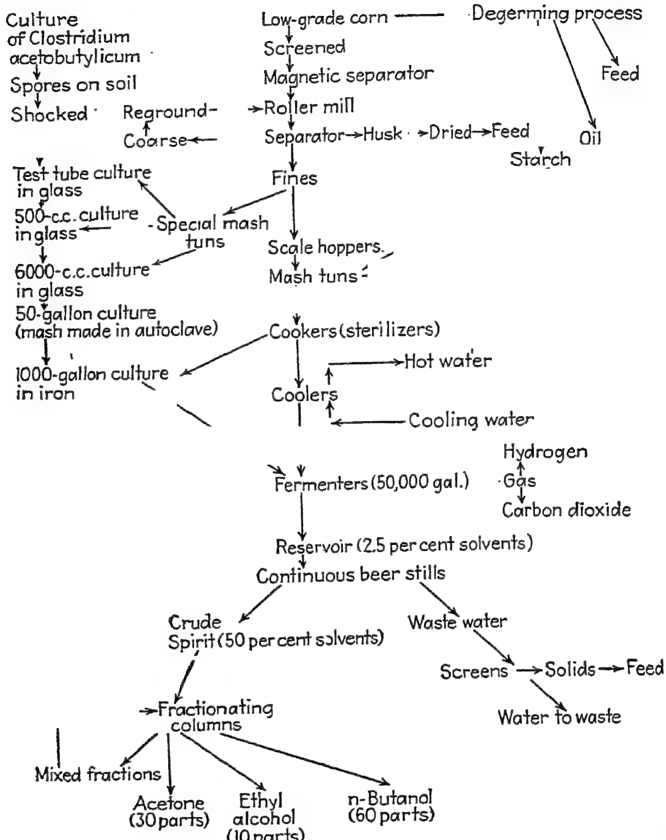


FIG. 18.—Flow sheet for butanol production. [*Courtesy D. H. Killeffer, Ind. Eng. Chem.* **19**: 46 (1927).]

There is, of course, a fairly large number of organisms, some non-sporeforming, which will produce butanol, but these bacteria would be unsatisfactory for commercial practice for various reasons. Some are

[1] "Bergey's Manual of Determinative Bacteriology," 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

pathogenic, some will produce only small quantities or traces of solvents, some are slow fermenters, some will not attack starch, some are aerobic, and some lack vigorous cultural characteristics.

Berthelot and Ossart[1] stated that of the many bacteria isolated by them, aerobic and facultative, which produced acetone, only a few produced it in a quantity that was at all comparable with that of the anaerobic organisms used in industry.

A cultural study of the acetone-butyl alcohol organism embodying such factors as reaction of the medium, temperature, reduction of dyes, and fermentability of carbohydrates was carried out by McCoy, Fred, Peterson, and Hastings.[2] They suggested *Cl. acetobutylicum* as a suitable name for the acetone-butanol organism of Weizmann.

Cl. acetobutylicum has now been adopted as the name of the species of *Clostridium* that produces relatively large quantities of acetone and butanol from starchy materials. Hence the Weizmann bacillus would properly be designated as *Cl. acetobutylicum* McCoy, Fred, Peterson, and Hastings.[3]

McCoy[4] and her associates have classified the motile and spore-forming butyric anaerobes of fermentation as a nonpathogenic subgroup of the genus *Clostridium,* which yields larger amounts of butyric acid or neutral products than the pathogenic clostridia, gives the granulose reaction and is catalase negative. The butyric anaerobes were divided into two general groups:

Group 1. Acid end products, chiefly butyric and acetic acids—the true butyric anaerobes.

Subtype *A*. *Cl. pasteurianum* type—nonstarch-fermenting bacteria.
Subtype *B*. *B. saccharobutyricus* type—starch-fermenting clostridia and occasionally plectridia.
Subtype *C*. Starch-fermenting plectridia.

Group 2. Butyric and acetic acids as intermediate products, followed by neutral products (alcohols, or alcohol plus acetone)—the butanol organisms of industry.

Culture Methods.—The continued transfer of a seed culture in the usual media results in the culture becoming sluggish, with a corresponding decreased yield of solvents. This observation has been made independently by several different workers.

[1] Berthelot A., et E. Ossart, *Compt. rend.,* **173**: 792 (1921).

[2] McCoy, E., E. B. Fred, W. H. Peterson and E. G. Hastings, *Jour. Infectious Diseases,* **39**: 457 (1926).

[3] "Bergey's Manual of Determinative Bacteriology," 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

[4] McCoy, Fred, Peterson, and Hastings, *op. cit.,* **46**: 118 (1930).

It has been shown that the most prodigious producers of solvents are those cultures whose spores are in general the most heat resistant. "Heat shocking" is a method wherein the vegetative cells and the weaker spores of a culture are destroyed. By subjecting a culture of the organism, in which the development of spores has been favored, to a temperature of 100°C. for 1 to 2 min., heat shocking is effected.

It must be borne in mind that the severity of the treatment bears a direct relation to the temperature employed, the size and nature of the tube containing the seed culture, and the characteristics of the medium. If one is to use thin-walled wafer tubing and a very small amount of the culture, it is obvious that neither the time of shocking nor the temperature should be excessive, since even the resistant spores may be destroyed.

Weyer and Rettger "pasteurized" their butanol cultures in capillary tubes, which were 5 cm. in length. The tubes were filled with saline suspensions of the spores and plunged into water at 100°C. An exposure of 45 sec. to this temperature was the limit of the tolerance of the spores. The capillary tubes were cooled quickly after the heat treatment.

Alternate heat shocking, or pasteurizing, and successive subculturing are commonly used to activate a culture. The medium containing the seed culture is allowed to stand at room temperature for a few days to encourage sporulation. New medium is inoculated from the spore-containing culture and heat shocked. The surviving spores are permitted to germinate under favorable conditions and subcultures are made successively at daily intervals for 4 to 7 days. At the end of this time the culture is again permitted to stand at room temperature to encourage spore formation. The cycle is then repeated—heat treatment, repeated subculturing, rest for sporulation, heat shocking, and so on.

Weizmann advocated heat treating a culture 100 to 150 times in order to improve its fermenting ability.

Underkofler and his coworkers[1] have shown that it is impossible to obtain good sporulation from sugar media when using ordinary culture tubes in the usual manner. They have demonstrated that sporulation could be successfully induced, however, provided the culture-containing medium was poured aseptically into a sterile flask of such size that the medium was exposed in a thin layer to air, or was dried on sterile soil.

Spores of *B. granulobacter pectinovorum,* grown in maize mash, retained their ability to produce acetone after storage for at least seven years in sealed glass tubes, according to Fowler and Subramanyan.[2] The spores were subcultured successfully in jawari mash (*Andropogon sorghum*).

[1] Underkofler, L. A., L. M. Christensen, and E. I. Fulmer, *Ind. Eng. Chem.,* **28**: 350 (1936).

[2] Fowler, G. J., V. Subramanyan, *Jour. Indian Inst. Sci.,* **8A**: 71 (1925).

In a study of six different strains of *Cl. acetobutylicum* (Weizmann), Weyer and Rettger (1927) brought out several facts of major importance. Storage of spores for a period of over 6 months apparently decreased their power of producing solvents. It was possible, however, to rejuvenate the cultures by alternate pasteurization and subculturing, for pasteurization destroyed the vegetative forms and the weaker spores. The most vigorous strains for fermentation appeared to be the most active sporeformers.

Cl. acetobutylicum (Weizmann) was rather susceptible to various antiseptic and germicidal substances. Spores withstood successfully the effects of a 2.5 per cent butyl resorcinol solution for 24 hr., but spores of *B. mesentericus* were destroyed or inactivated. The apparently selective action of the butyl resorcinol was believed to be due to the fact that the Weizmann organism has a certain tolerance for the butyl radicle.

Raw Materials.—A rather wide variety of raw materials may be used to supply the carbohydrates and nutrient substances required to ensure a satisfactory fermentation. Starches rendered soluble by preliminary treatment, hydrolyzed starches, disaccharides, hexoses, pentoses, molasses, and sugar sirups have been used under different conditions. Corn is, of course, a readily available and easily fermented source of raw material in this country and elsewhere. Rice, jawari, bajra, and tapioca starches; peanut and oat hulls; corncobs; horse chestnuts; arabinose; and xylose have been fermented by butanol organisms with varying degrees of success. Underkofler and others[1] have shown experimentally that as much as 80 per cent of corn meal may be replaced by sucrose or glucose without sacrificing high yields. Xylose may replace corn meal to the extent of 40 per cent. Thus it is possible to ferment along with corn meal materials which, by themselves, would produce small yields or be fermented with some difficulty. This is a sound practice from the point of view of conservation and economics.

Sjolander and his coworkers[2] have shown that butanol, acetone, and ethanol may be successfully produced from wood sugars by *Cl. felsineum* and *Cl. butylicum*.

Nitrogen Requirements.—According to Wilson and his associates,[3] *Cl. acetobutylicum* was able to utilize protein, peptone, or aminoids (completely degraded proteins) as sources of nitrogen.

There were only slight changes in the ratio of solvents resultant from the use of nitrogen in the different forms. When peptone was the source of nitrogen, more acetone was produced with less ethanol; when beef

[1] Underkofler, Christensen, and Fulmer, *loc. cit.*

[2] Sjolander, N. O., A. F. Langlykke, and W. H. Peterson, Butyl Alcohol Fermentations of Wood Sugar, *Ind. Eng. Chem.*, **30**: 1251 (1938).

[3] Wilson, P. W., W. H. Peterson, and E. B. Fred, *Jour. Bact.*, **19**: 231 (1930).

aminoids were used, there was a small increase in the quantity of butanol. Large amounts of ammonium salts of the mineral acids prevented fermentation or decreased yields on account of the acids liberated upon hydrolysis. Mashes containing a deficiency of nitrogen were decidedly abnormal from the point of view of yield of solvents.

Ammonium salts or single amino acids do not serve as satisfactory sources of nitrogen in semisynthetic media.

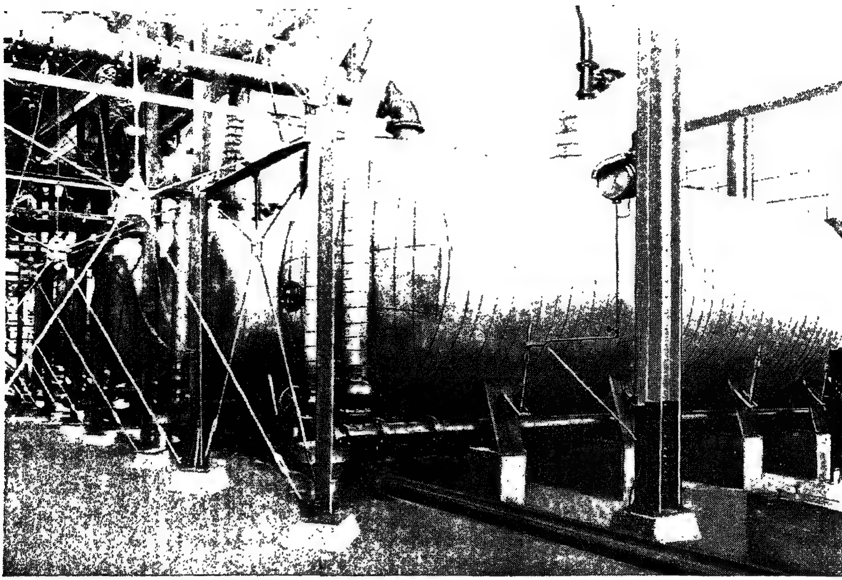


FIG. 19.—Cookers for sterilizing the corn meal and water. (*Courtesy G. O. Lines, Commercial Solvents Corp.*)

Some Substances Essential for Normal Yields of Solvents.—Speakman, working with *B. granulobacter pectinovorum,* found that no growth resulted when the sole source of nitrogen in a mash was ammonium salts or a single amino acid. Weyer and Rettger[1] substantiated Speakman's conclusions and stated that a complex nitrogen supply, which may be furnished by proteins and commercial peptones, is necessary for growth of *Cl. acetobutylicum* and subsequent fermentation.

Working with *Cl. acetobutylicum* (Weizmann), Weinstein and Rettger[2] found that a prolamine-containing substance was necessary for the production of normal amounts of acetone and butanol by fermentation.

[1] WEYER, E. R., and L. F. RETTGER, *Jour. Bact.*, **14**: 399 (1927).

[2] WEINSTEIN, L., and L. F. RETTGER, *Jour. Bact.*, **23**: 74 (1932).

Hydrolyzed cottonseed hulls, peanut hulls, corncobs, and the like yielded normal amounts of acetone but no butanol. The addition of a prolamine-containing substance, yellow corn, stimulated the production of normal amounts of both solvents. The yields of solvents were higher than those obtained from corn alone or from the hydrolyzed product alone. It was ascertained that prolamine did not act as a catalyst. An alcohol-soluble protein was found to be necessary for the production of appreciable amounts of butanol from Robinson's medium.

On the other hand, Weizmann and Rosenfeld (1937) state that complex proteins, such as peptone or prolamines, are not necessary for a normal butanol-acetone fermentation. They have shown that asparagine in the presence of an activator will produce a normal fermentation in a semisynthetic medium. The activator is a compound of low molecular weight of unknown composition. It is possibly not a single substance. According to Weizmann, the activator is probably of the nature of a coenzyme, which may play an essential part as a hydrogen carrier as well as favor growth of bacteria. Insufficient anaerobiosis, absence of the activator, or both, led to the production of acids, principally butyric acid. In the absence of asparagine and the activator, the fermentation may not proceed at all, or very slowly. The activator is found in seeds, green plants, and in yeast. It is thermostable. (For a further discussion of this subject, the reader is referred to the publication by Weizmann and his associate.)

Brown and his associates[1] obtained normal yields of neutral solvents by culturing butanol-acetone organisms in a medium that contained glucose, hydrolyzed casein, tryptophane, ammonium sulphate, mineral salts, and an "acidic ether-soluble extract" obtained from Difco yeast extract.

McDaniel, Woolley, and Peterson[2] have partially purified an accessory substance, which together with glucose, asparagine, and inorganic salts permitted growth of *Cl. acetobutylicum* and of *Cl. butylicum* in a medium. The stimulating substance could not be replaced by beta-alanine, indole acetic acid, inositol, nicotinic acid amide, pantothenic acid, pimelic acid, riboflavine, sporogenes growth factor, vitamin B_1, vitamin B_6, or by a "mixture of all of the naturally occurring amino acids" (hydroxy-glutamic acid excepted). It was stable to bromination, to steaming in normal alkali, and to autoclaving.[3]

Substances Stimulating the Production of Butanol by Certain Butyric Acid Bacteria.—In 1934, Tatum, Peterson, and Fred[4] reported the pres-

[1] Brown, R. W., H. G. Wood, and C. H. Werkman, *Jour. Bact.*, **35**: 206 (1938).

[2] McDaniel, L. E., D. W. Woolley, and W. H. Peterson, *Jour. Bact.*, **37**: 259 (1939).

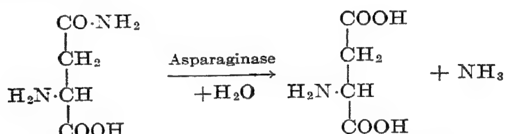
[3] *Ibid.*

[4] Tatum, E. L., W. H. Peterson, and E. B. Fred, *Jour. Bact.*, **27**: 207 (1934).

ence of an unknown substance in potatoes, yams, cabbage, lettuce, alfalfa, navy and soybeans, malt sprouts, and wheat middlings that caused an increased fermentation of starch and a marked increase in the yield of butanol through the action of certain butyric acid bacteria. No changes were effected in the quantities of ethyl alcohol and acetone produced. Barley, corn, oats, and rice were poor sources of the stimulating substance and sometimes were found to contain none of it.

Later, Tatum and his associates[1] identified *l*-asparagine ($H_2NOC \cdot CH_2 \cdot CH(NH_2) \cdot COOH$) as the substance producing the stimulatory action. Further research disclosed that *l*-aspartic ($HOOC \cdot CH_2 \cdot CH(NH_2) \cdot COOH$) and *d*-glutamic ($HOOC \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$) acids, which are dicarboxylic amino acids, produced effects equivalent to those of *l*-asparagine when these acids were supplemented with molar equivalents of ammonium sulphate. The optimum concentration of these stimulants was 60 mg. per 100 cc. of 4 per cent corn-meal mash, although lower concentrations produced stimulation.

Asparagine may be hydrolyzed by the enzyme asparaginase to yield aspartic acid and ammonia.



Asparagine Aspartic acid

Asparaginase is secreted by some yeasts and bacteria and has an optimum pH of 8.

Ammonium malate ($H_4NOOC \cdot CH_2 \cdot CHOH \cdot COONH_4$) and ammonium succinate ($H_4NOOC \cdot CH_2 \cdot CH_2 \cdot COONH_4$) were likewise found to stimulate the fermentation of starch by certain, but not all, butyric acid bacteria and to cause increases in the yields of butanol, but these substances were less effective than *l*-asparagine.

Optimum Conditions for Fermentation. *Temperature.*—The most favorable temperature range is 37 to 42°C. Since the neutral solvents are volatile at these temperatures, especially acetone, it is necessary to take precautions to avoid losses during the fermentation process.

Oxygen Relationship.—Since the organisms best suited to the production of acetone and butanol are anaerobic in nature, the highest yields will be obtained when anaerobiosis is maintained.

pH.—Growth may be obtained in corn mashes between a pH of 4.7 and 8 by the butanol organisms, but there is a low production of solvents at both of the extremes. The pH range of 5 to 7 is in most cases satisfactory.

l., **29**: 563 (1935).

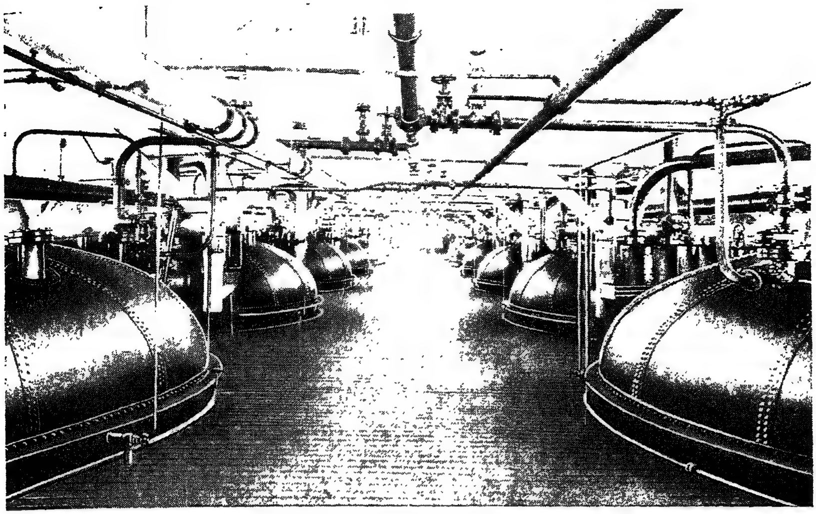


FIG. 20.—Upper level of 50,000-gal. fermentation tanks. (*Courtesy G. O. Lines, Commercial Solvents Corp.*)

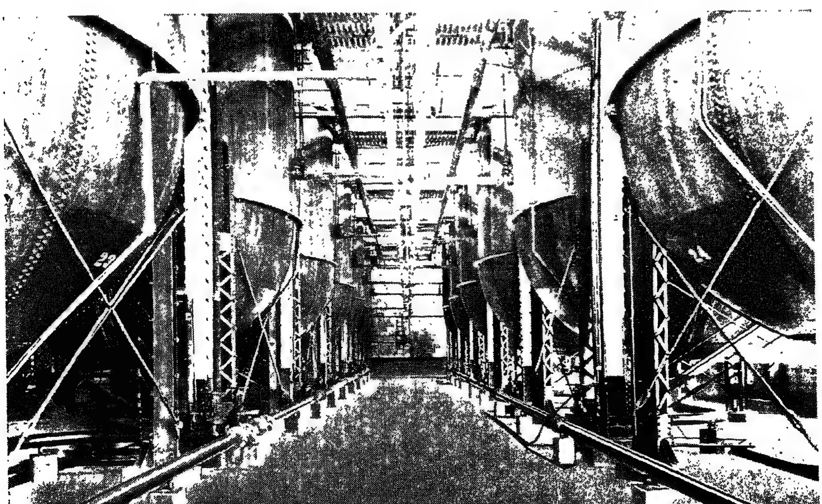


FIG. 21.—Lower level of 50,000-gal. fermentation tanks. (*Courtesy G. O. Lines, Commercial Solvents Corp.*)

The assertion that a given concentration of hydrogen ions is completely inhibitory at all times or is capable of producing any given degree of inhibition, is not justified, according to Wynne,[1] who carried out experiments upon the inhibition of the acetone-butanol fermentation by acids. The inhibition of fermentation was in the following order: nonylic $\geqq$ caprylic > heptylic > formic > isocaproic = caproic > valeric = isovaleric > isobutyric = butyric $\geqq$ propionic = acetic. This order is similar to that of the speed of penetration of the acids into the living

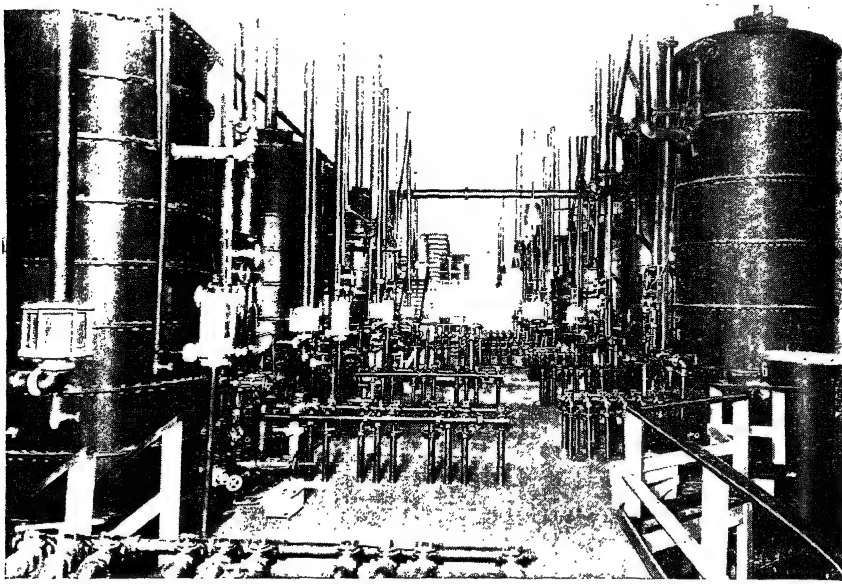


FIG. 22.—Distillation equipment. (*Courtesy G. O. Lines, Commercial Solvents Corp.*)

cells. The pH necessary for pyruvic acid to inhibit is 3.2 which is lower than for most acids.

Effect of Calcium Carbonate.—In general, the addition of calcium carbonate to the acetone-butanol fermentation medium causes a decrease in the yields of acetone and butanol in proportion to the amount of calcium carbonate added. The volatile acid content, butyric and acetic acids, principally, is increased, while alcohol formation is suppressed.

Concentration of Raw Material.—The exact concentration of the carbohydrate-containing substance to be used in a given fermentation must be determined by experimentation, unless facts concerning the fermenta-

[1] WYNNE, A. M., *Jour. Bact.*, **22**: 209 (1931).

tion organism are already known. Mashes containing 3 to 10 per cent concentrations of corn meal are readily fermented with high yields.

Yield of End Products.—In the normal butanol-acetone fermentation, neutral solvents are formed from glucose in the ratio of 6 parts of *n*-butanol, 3 parts of acetone, and 1 part of ethyl alcohol. Sucrose, levulose, and xylose yield normal ratios of butanol, acetone, and ethanol, but arabinose, a 5-carbon sugar, yields the solvents in a ratio of approximately 5:4:1 instead of the normal 6:3:1 ratio. These facts are illustrated in the following table.

TABLE 42.—AVERAGE SOLVENT RATIO FOR THE ACETONE-BUTANOL FERMENTATION OF SUGARS[1]

Investigators	Sugar	No. of fermentations	Average solvent ratio		
			Butanol	Acetone	Ethanol
Peterson, Fred, and	Glucose	2	59	31	10
Schmidt[2]	Xylose	3	61	29	10
	Arabinose	2	47	43	10
Johnson, Peterson,	Glucose	18	60	28	12
and Fred	Arabinose	4	48	39	13

[1] UNDERKOFLER, L. A., and J. E. HUNTER, JR., *Ind. Eng. Chem.*, **30**: 480 (1938).
[2] Ratios calculated from the data, assuming 10 per cent ethanol.

From 3 lb. of starch, 1 lb. of mixed solvents may be obtained when *Cl. acetobutylicum* is used as the fermentation organism.

At the Royal Naval Cordite Factory at Holton Heath, England, 163 lb. of *n*-butanol, 70 lb. of acetone, 407 lb. of carbon dioxide, 11 lb. of hydrogen, and 12 lb. of residual acid were obtained from 1,000 lb. of maize, which contained 650 lb. of starch.[1]

The gases produced during fermentation weigh over 1.5 times as much as the neutral solvents formed. Were these gases allowed to go to waste, as they were at one time, the losses would be great.

Acetylmethylcarbinol.—In 1927, Wilson and his associates[2] showed that acetylmethylcarbinol was produced in the acetone-butanol fermentation by *C. acetobutylicum* (Weizmann's bacillus) as a regular end product. Acetylmethylcarbinol was formed concurrently with acetic and butyric acids. Pyruvic acid when added to a fermenting mash was fermented to acetic acid, acetone, and acetylmethylcarbinol chiefly (Peterson and Johnson, 1933). The addition of phosphates to a mash increased the production of acetylmethylcarbinol, while added proteins decreased the

[1] REILLY, HICKENBOTTOM, HENLEY, and THAYSEN, *loc. cit.*
[2] WILSON, P. W., W. H. PETERSON, and E. B. FRED, *Jour. Biol. Chem.*, **74**: 495 (1927).

TABLE 43.—CHEMICALS FROM CORN AND THEIR USES[1]

ACETONE		CORN OIL / CORN GERM MEAL	BUTANOL		ETHYL ALCOHOL / CARBON DIOXIDE	METHANOL
ACETYLENE SOLVENT	PHOTOGRAPHIC FILMS	LACQUERS	PHOTOGRAPHIC FILMS	METAL CLEANERS		
CELLULOSE ACETATE SILK	PYROXYLIN PLASTICS	SPIRIT VARNISHES	ARTIFICIAL AND PATENT LEATHER	ALCOHOL DENATURANT	SYNTHETIC RESINS	PAINT & VARNISH REMOVER
LINIMENTS	CELLULOSE ACETATE	DYE SOLVENT	DRUG EXTRACTION	DEHYDRATER	EMBALMING FLUID	DYE SOLVENT
AIRPLANE DOPES	NITROCELLULOSE CEMENTS	PENETRATING OILS	SOLDERING FLUX	DE-FROTHER	ANTIFREEZE	
PAINT & VARNISH REMOVER	DEWAXING OILS	RUST REMOVERS	SYNTHETIC RESINS	DRY CLEANING	SPIRIT VARNISHES	DEWAXING GUMS
ALCOHOL DENATURANT	ARTIFICIAL LEATHER	BUTYL PROPIONATE	BUTYL XANTHATE	BUTYL CELLOSOLVE	ESSENTIAL OIL SOLVENT	DRY CLEANING
SYNTHETIC RESINS	SMOKELESS POWDER				LITHOGRAPHIC WORK	PYROXYLIN PLASTICS
EXTRACTION	DRUGS	RUBBER ACCELERATOR	LACQUERS	PLASTICIZER	METHYL ANTHRANILATE	METHYL CHLORIDE
CHLOROFORM	IODOFORM	BUTYL CHLORIDE	BUTYL STEARATE	BUTYL LACTATE	DIMETHYL ANILINE	DIMETHYL AMINE
DIACETONE ALCOHOL		ALCOHOL DENATURANT	WATERPROOFING COMPOUNDS	BRUSHING LACQUERS	NOMETHYL AMINE	
ANTIFREEZE	COMPRESSION FLUIDS				DEHAIRING HIDES	DYE INTERMEDIATE
			ATE IER	EMULSIFICATION AGENT		

[1] Courtesy Commercial Solvents Corp.

yield. Ordinarily 300 to 400 mg. per liter of acetylmethylcarbinol are produced in the butanol fermentation.

Yellow Oil.—In the butanol fermentation, so-called "yellow oil" makes up 0.5 to 1 per cent of the total yield of solvents. Marvel and Broderick[1] showed that the high boiling yellow oil was a complex mixture of *n*-butyl alcohol, active amyl alcohol, isoamyl alcohol, *n*-hexyl alcohol, and the *n*-butyric, caprylic, and capric esters of these alcohols.

Uses for the Products of Fermentation.—Butanol has its most important use in the manufacture of lacquers, which are utilized on automobiles, airplanes, furniture, toys, and many other articles. Large quantities of butanol and its derivatives are used in other industrial processes. Acetone is used in the manufacture of artificial silk and leather, photographic film, airplane dopes, cements, and other products. The accompanying table shows some detailed uses for the chemical products manufactured from corn.

The weight of gases evolved during fermentation is over 1½ times greater than the weight of solvents. Hence, in order to prevent enormous wastes, uses for the hydrogen and carbon dioxide gases have been developed. Methanol (CH_3OH) is synthesized from hydrogen and carbon dioxide gases. These gases are passed through a solvent recovery plant to remove and recover any solvents that may have been carried over with the gases during the fermentation. Part of the carbon dioxide is then removed by "scrubbing" the gases with water under pressure. The purified gases are then forced through a porous, catalytic mixture at a high temperature and at a pressure of about 4,500 lb. to produce synthetic methanol. Ammonia may be synthesized from purified hydrogen and nitrogen gases, using a catalyst, while carbon dioxide may be used in dry-ice manufacture.

Bacterial Contaminants.—The most serious contaminants in the acetone-butanol fermentation are the lactic acid organisms. Lactic acid bacteria grow readily at the temperature of the butanol fermentation and under anaerobic conditions. They utilize the substrate and at the same time produce a pH unfavorable for butanol production. The high-acid-forming bacteria,[2] such as *Lactobacillus leichmannii*, are most injurious. Organisms such as *L. mannitopoeum* are likewise very undesirable contaminants. *B. volutans* n. sp. Fleming, Thaysen,[3] a most serious contaminant, is a nonsporeforming organism that produces large amounts of lactic acid, traces of butyric and acetic acids, but no gas or alcohols. It is a Gram-positive organism, possessing volutin granules that may be stained by methylene blue to a deep purple. This organism may be

[1] Marvel, C. S., and A. E. Broderick, *Jour. Am. Chem. Soc.*, **47**: 3045 (1925).
[2] Fred, E. B., W. H. Peterson, and M. Mulvania, *Jour. Bact.*, **11**: 323 (1936).
[3] Thaysen, A. C., *Jour. Inst. Brewing*, **27**: 529 (1921).

destroyed by heating it for 5 min. at 65°C. *Streptococcus lactis* also has been known to cause infections, but it does not usually produce serious trouble. Fermentations continue to completion unless the organisms are present in large numbers in the mash before the butanol organisms have had opportunity to develop.

The presence of *B. globigii* in a butanol fermentation is a type of association that produces no apparent ill effect on the yield by *B. granulobacter*.[1]

Sporeformers of the *B. mesentericus* group may produce a red pigment in corn mashes[1] but otherwise produce no apparent harm.

Methods of Detecting Contamination.—Various methods have been used to detect contamination in the fermenting mash.[2] Observation of variations in gas evolution or in the titrable-acidity curve, and the use of the microscope are the most common methods.

Perhaps the most sensitive indicator of contamination is gas evolution. One accustomed to observing normal fermentations can readily ascertain irregularities in the evolution of gas. A marked reduction in the volume of gas evolved at a time when the rate of evolution should be increasing may be taken as an indication of contamination. Gas evolution may even cease.

By plotting the curve for titrable acidity, one has available accurate information concerning the progress of the fermentation. Any increase in acidity to abnormal amounts can be readily ascertained by an examination of the acid curve. After rising to a peak in 13 to 17 hr., the normal curve for titrable acidity drops at about the same rate to a new low point. In case the mash is contaminated by lactic acid organisms, the curve for titrable acidity continues to rise after the normal maximum instead of returning to the new low point. In some industrial plants a practice was made to inoculate large mashes from starters only after the curve for titrable acidity had commenced to fall. (The reader should realize that variations in the nature of the curve for titrable acidity sometimes occur even though there is an absence of infection.)

The microscope has served as a useful tool in detecting contamination, yet it may fail to show contamination when either variations in gas evolution or titrable acidity may strongly indicate the presence of infective organisms. The microscope is best used in conjunction with one of the foregoing methods rather than alone.

Marked changes in the curve for pH would indicate unusual conditions in the fermenting mash. Changes in pH do not, however, furnish either as sensitive or as accurate signs of an abnormal fermentation as do abnormal variations in gas evolution and in titrable-acidity curves.

[1] Fred, E. B., W. H. Peterson, and W. R. Carroll, *Jour. Bact.*, **10**: 97 (1925).

[2] Speakman, H. B., and J. F. Phillips, *Jour. Bact.*, **9**: 183 (1924).

Biochemistry of the Fermentation.—Speakman[1] divides the butanol fermentation into three phases, the length of each phase being governed by the curve for titrable acidity. During the first phase, the titrable acidity increases rapidly to a maximum, usually in 13 to 17 hr. The butanol organisms reproduce very rapidly during this period, for it is their enzymic activity that determines the progress of the fermentation. Acetic and butyric acids are produced in varying quantities. Both hydrogen and carbon dioxide gases are produced in large amounts, the curve for total gas evolution following the titrable-acidity curve in general proportions but with a time lag. There is a drop in pH, which

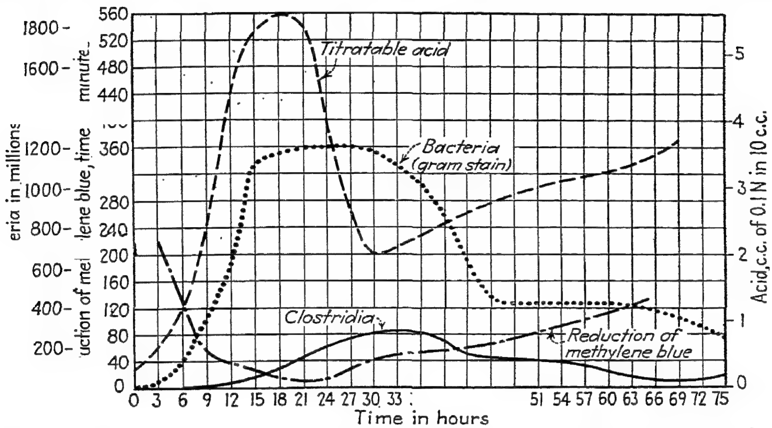


FIG. 23.—Number of bacteria, time of reduction of methylene blue, and acid production at different stages during fermentation. [*Courtesy W. H. Peterson and E. B. Fred, Ind. Eng. Chem.* **24**: 237 (1932).]

then tends to remain at a fairly constant level throughout the rest of the fermentation, owing to the presence of buffers produced through the hydrolysis of the corn or other proteins.

The quantity of titrable acidity drops sharply during the second phase of fermentation to a value that is equal to approximately 50 per cent of the maximum. Coincidental with the drop in the quantity of titrable acidity, there is a rapid conversion of the acids to their corresponding solvents. Butyric acid is reduced to butanol, acetic acid is changed to acetone. The ratio of acetic to butyric acid varies during this period. Butyric acid disappears from the fermentation mash more rapidly than acetic acid. The rate of gas evolution increases quickly to a maximum as the titrable acidity drops from the peak. There is then a gradual diminution in the rate of gas evolution until the end of the fermentation.

[1] SPEAKMAN, H. B., *Jour. Biol. Chem.*, **41**: 319 (1920).

The titrable acidity slowly increases in quantity during the third phase of the fermentation. There is a drop in the rate of solvent production until the fermentation ceases. The relative proportions of acetic and butyric acids continue to vary until at the end of the fermentation there is a greater amount of acetic acid.

Peterson and Fred[1] have carried out extensive research on the bacteriology and biochemistry of the acetone-butanol fermentation by *Cl. acetobutylicum.* Figures 23, 24, 25, 26, 27, and 28 are reproduced

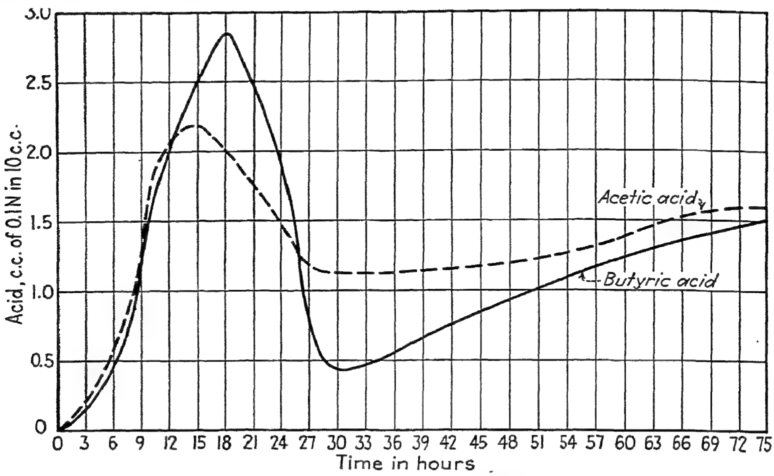


FIG. 24.—Nature of volatile acids formed by *Cl. acetobutylicum.* [*Courtesy W. H. Peterson and E. B. Fred, Ind. Eng. Chem.* **24**: 237 (1932).]

(through their courtesy) from their excellent paper. The data from which the curves were constructed were determined by periodic analyses of the fermenting corn mashes, which at the start were of 6 per cent concentration. Results shown in Figs. 23 to 26 were obtained in the first experiment of Peterson and Fred, those of Fig. 27 in the second experiment, and those of Fig. 28 in the third experiment. A study of these figures will yield much valuable information.

Only small amounts of peptides or amino acids are formed from corn mashes during the first 12 hr. of the fermentation and not much during the first 24 hr., according to Peterson and his associates.[2] The amino acid and peptide content of the mash increases rapidly during the next 24 hr., however.

[1] PETERSON, W. H., and E. B. FRED, *Ind. Eng. Chem.*, **24**: 237 (1932).

[2] PETERSON, W. H., E. B. FRED, and B. P. DOMOGALLA, *Jour. Am. Chem. Soc.*, **46**: 2086 (1924).

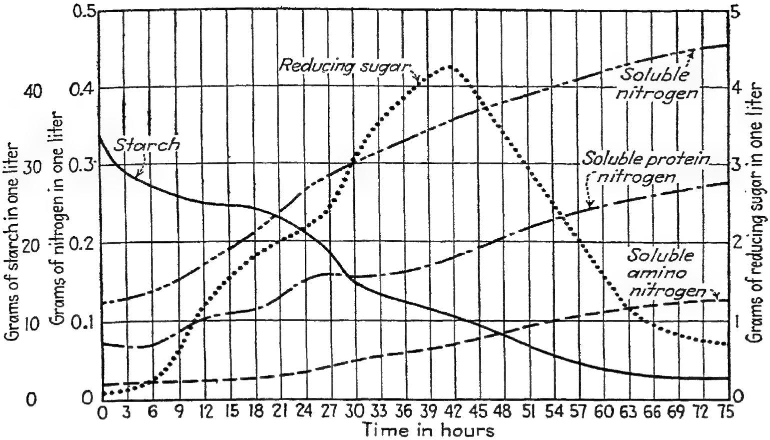


FIG. 25.—Changes in carbohydrates and forms of nitrogen caused by *Cl. acetobutylicum*. [*Courtesy W. H. Peterson and E. B. Fred, Ind. Eng. Chem.* **24**: 237 (1932).]

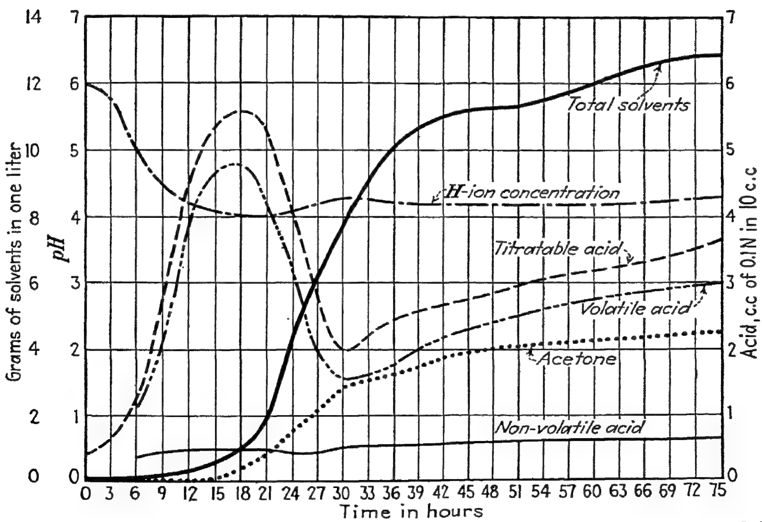


FIG. 26.—Acid production and solvent formation. [*Courtesy W. H. Peterson and E. B. Fred, Ind. Eng. Chem.* **24**: 237 (1932).]

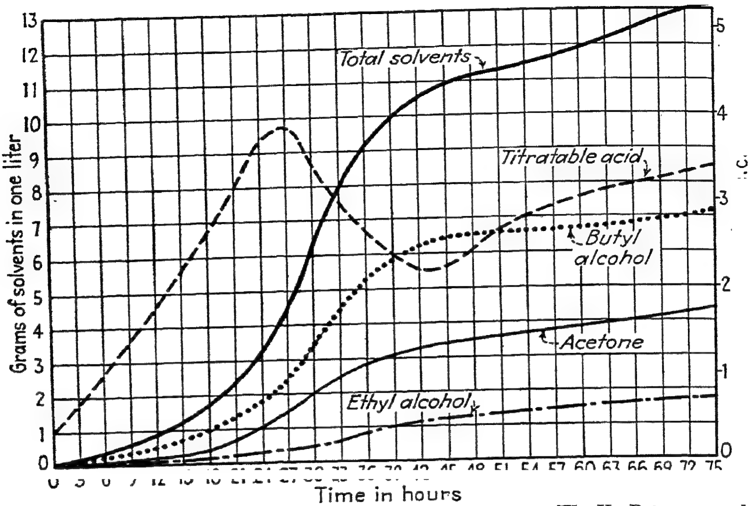


FIG. 27.—Acid production and solvent formation. [*Courtesy W. H. Peterson and E. B. Fred, Ind. Eng. Chem.* **24**: 237 (1932).]

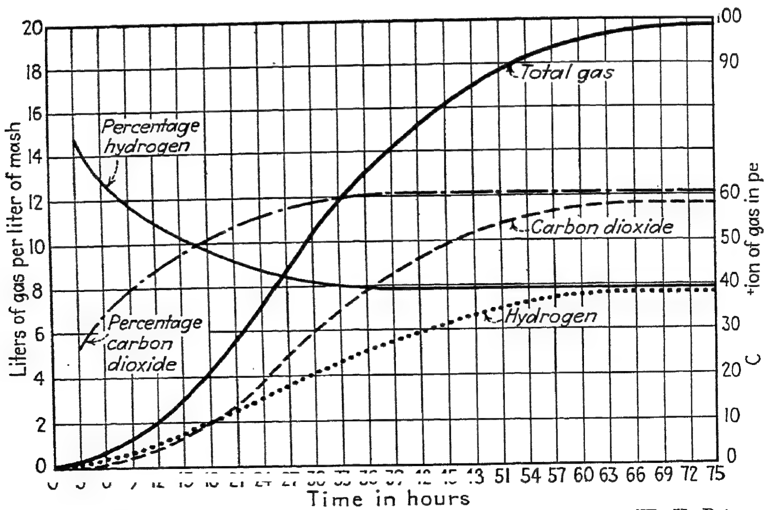


FIG. 28.—Production of gas in butyl alcohol fermentation. [*Courtesy W. H. Peterson and E. B. Fred, Ind. Eng. Chem.* **24**: 237 (1932).]

Fulton, Peterson, and Fred[1] state that from 15 to 60 per cent of the total nitrogen of native proteins is rendered soluble during the fermentation. The soluble derivatives of protein (proteoses, peptones, peptides, and amino acids) and nonamino nitrogen constitute the soluble nitrogen. Peptide nitrogen is usually the largest single source of soluble nitrogen, constituting 9 to 28 per cent of the total. Amino nitrogen is generally present in quantities of 7 to 18 per cent, depending upon the type of protein being fermented, animal proteins yielding rather high quantities of amino nitrogen.

The products formed by the hydrolysis of proteins, a process that appears to progress well at a pH of about 5.5, regulate the pH of the medium through buffer action. Butyric and acetic acids are but slightly dissociated. Thus high titrable acidity produces very little change in the pH of the medium.

Factors that cause an increase in acetone frequently produce a decrease in the quantity of ethanol formed. Proteolysis, acid production, and the quantity of solvents produced are affected by the carbohydrate-protein ratio of the mash. Low carbohydrate-protein ratios favor increased yields of acetone and decreased yields of ethanol. Opposite effects result from high carbohydrate-protein ratios. In general, high yields of solvents may be obtained with carbohydrate-protein ratios of 5 to 10 (Fulton, Peterson, and Fred, 1926). High titrable acidity is generally associated with a high yield of acetone.

Peterson and Johnson[2] found that *Cl. acetobutylicum* fermented added acetoacetic acid most rapidly during the period when solvents were being formed at a maximum rate. As much as 3.2 g. of acetoacetic acid per liter has been fermented during 8 hr. in some fermentations, the acid being decarboxylated to acetone.

Formic acid added to a fermenting mash was destroyed by *Cl. acetobutylicum*.[3] This fact strengthens the hypothesis that formic acid is the precursor of hydrogen and some of the carbon dioxide gas.

For a further discussion of the biochemistry of the butanol fermentation, the reader is referred to the section dealing with the mechanism of the fermentation.

Mechanism for the Formation of End Products.—Before attempting to formulate a scheme to show the origin of the products of a fermentation, it is necessary to assemble some of the known facts. A careful

n, H. L., W. H. Peterson, and E. B. Fred, *Centr. Bakt. Parasitenk.*, Abt. II, **27**: 1 (1926).

[2] Peterson, W. H., and M. J. Johnson, *Jour. Bact.*, **25**: 69 (1933).

[3] Stiles, H. R., W. H. Peterson, and E. B. Fred, *Jour. Biol. Chem.*, **84**: 437 (1929).

study of the charts concerned with the biochemistry of the butanol fermentation will be most fruitful in this connection.

During the butanol-acetone fermentation, butyric acid disappears more rapidly than acetic acid, a fact in accord with the production of a greater proportion of butanol than acetone (Reilly, Hickinbottom, Henley, and Thaysen). The decrease in butyric acid is coincidental with the increase in butanol. Additions of butyric acid to a fermenting mash increase the yield of butanol (Speakman and others). Butyric acid is nearly always found in fermentations when butanol occurs.

Acetic acid disappears from the fermentation medium more slowly than butyric acid, a fact agreeing with the formation of a smaller amount of acetone than butanol. The addition of acetic acid to a fermenting mash increases the acetone content, but, according to Speakman, does not cause any change in the yield of butanol. Acetic acid, acetoacetic acid, and pyruvic acid increase the quantity of acetone produced when added to the fermentation (Johnson, Peterson, and Fred, 1933). Propionic acid is reduced to propyl alcohol.

Pyruvic acid is fermented to acetic acid, acetone, and acetylmethylcarbinol when added to the fermented mash, but methylglyoxal and aldol are toxic even in small amounts.

Calcium carbonate in the fermentation mash results in a suppression of butanol and acetone production but causes an increase in the quantities of butyric and acetic acids.

A workable scheme must show the derivation of all the end products in relatively correct proportions. It must be in accord with established facts of fermentation chemistry. Study of the following schemes will show how closely these principles are approached.

Several schemes have been suggested to explain the origin of the end products of a normal butanol-acetone fermentation. Fitz suggested the formation of 4-carbon compounds from 2-carbon compounds by a process of condensation.

Speakman[1] was one of the first to suggest a detailed scheme for the mechanism of the butanol-acetone fermentation. According to him butyric and acetic acids were formed by cleavages and oxidations of the sugar molecule. These acids were then reduced in part to the corresponding solvents.

Neuberg and Arinstein[2] suggested that butyric acid and butanol were formed from glucose and glycerol through the intermediate stages of pyruvic acid and pyruvic aldol.

[1] Speakman, H. B., *Jour. Biol. Chem.*, **58**: 395 (1923).

[2] Stephenson, M., "Bacterial Metabolism," Longmans, Green & Company, New York, 1930.

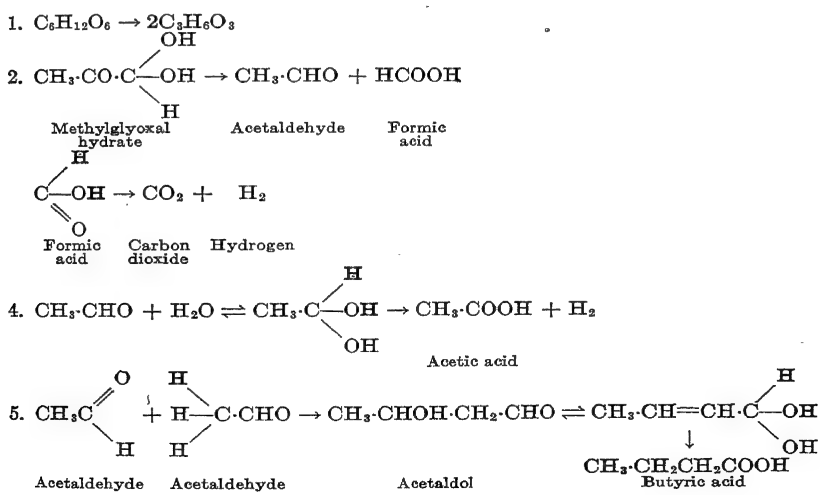
Pyruvic acid is produced by fermentation from glucose, glycerol, or lactic acid by various microorganisms. But Neuberg and Arinstein were unable to increase the yields of butyric acid or butanol through the addition of a pyruvate to a fermenting medium. They did, however, secure increases of acetic and formic acids in accordance with the following equation:

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + \underset{\text{Formic acid}}{HCOOH}$$

Neuberg and Arinstein therefore concluded that butyric acid and butanol did not arise as a result of the condensation of acetaldehyde and carbon dioxide. The addition of pyruvic aldol resulted in increased yields of butyric acid. On the basis of these facts, they formulated their schemes.

Various objections have been raised in connection with the schemes suggested by Neuberg and Arinstein. The organism that they used, *B. butylicus*, under optimum conditions produced very little butanol. The fact that added pyruvic aldol was fermented to butyric acid does not prove that it was an intermediate product. Even though acetaldehyde was not produced from pyruvic acid, this fact cannot be taken as proof that it does not arise from some other source.

Kluyver[1] and his associates have formulated a scheme for the butyric acid fermentation, which follows:



[1] Kluyver, A. J., "The Chemical Activities of Micro-organisms," University of London Press, Ltd., 1931.

In the butyric acid fermentation, the main end products are butyric acid, acetic acid, carbon dioxide, hydrogen, and traces of formic acid.

Kluyver is of the opinion that the mechanism for the conversion of sugar to hydrated methylglyoxal in the butyric acid fermentation is analogous to that of the alcoholic fermentation.

According to the scheme shown above, the weight of the carbon dioxide formed should be equivalent to approximately one-half of the weight of the glucose fermented. (Compare with the ethanol fermentation.) The number of molecules of acetaldehyde required for the formation of acetic acid and butyric acid should be equivalent to the number of molecules of carbon dioxide produced. Also the total number of molecules of hydrogen gas evolved (Eqs. 3 and 4) should be greater than the number of molecules of acetic acid produced (Eq. 4) by the number of molecules of carbon dioxide evolved. The following table indicates that these requirements are reasonably satisfied.

TABLE 44.—FERMENTATION BALANCE OF GLUCOSE, USING *Clostridium saccharobutylicum*[1]

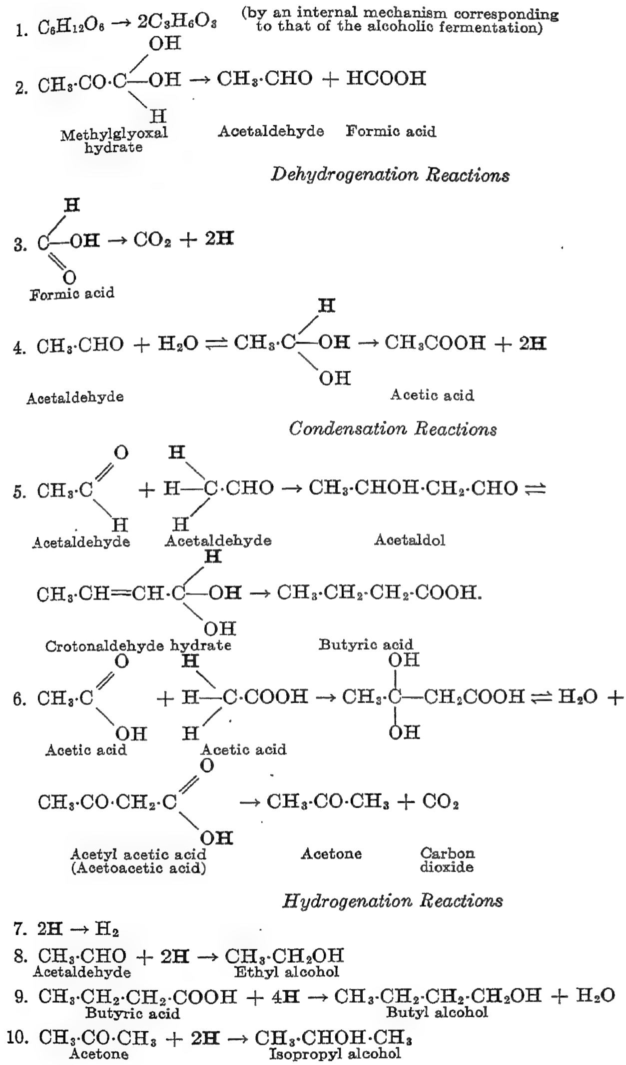
(Medium: yeast extract, 2 per cent glucose, 1 per cent calcium carbonate, 30°C.)

Products	Grams	Per cent of glucose fermented	No. of mols per 50 mols of glucose fermented		
			CO_2	H_2	Acetaldehyde
Glucose added.......	39.45				
Glucose unfermented.	3.10				
Glucose fermented...	36.35				
Carbon dioxide......	17.4	47.8	97.8		
Hydrogen...........	0.94	2.59		116.6	
Formic acid.........	Traces				
Acetic acid..........	5.17	14.2		−21.3	21.3
Butyric acid.........	13.4	36.9			75.5
Total.............			97.8	95.3	

[1] KLUYVER, A. J., "The Chemical Activities of Micro-organisms," University of London Press, Ltd., 1931.

It is necessary to account for several additional products when one turns from the butyric acid to the butanol-acetone fermentation. A scheme for this fermentation, to be acceptable, must satisfy conditions in respect to the relative proportions of each of the nine or more products found in the breakdown of a sugar. The scheme suggested by Kluyver does this well according to the results of Tables 45 and 46, one of which gives data for a medium containing 2 per cent calcium carbonate.

SCHEME FOR THE BUTYL ALCOHOL FERMENTATION[1]



[1] KLUYVER, A. J., "The Chemical Activities of Micro-organisms," University of London Press, Ltd., 1931.

VAN DER LEK, J. B., "Onderzoekingen over de Butylalkoholgisting," Naamlooze Vennootschap W. D. Meinema, Delft, 1930.

TABLE 45.—FERMENTATION BALANCE OF GLUCOSE, USING *Cl. acetobutylicum*[1]
(Medium: yeast extract, 2 per cent glucose, 37°C.)

Products	Grams	Per cent of glucose fermented	Mols per 50 mols of glucose fermented		
			CO_2	H_2	Acetaldehyde
Glucose added	11.78				
Glucose unfermented	0.41				
Glucose fermented	11.37				
Carbon dioxide	6.14	54.0	110.3		
Hydrogen	0.1770	1.5		70.2	
Formic acid	Traces				
Acetic acid	0.53	4.7		−7.0	7.0
Butyric acid	0.24	2.1			4.2
Ethyl alcohol	0.27	2.4		4.7	4.7
Butyl alcohol	2.62	23.0		56.0	56.0
Acetone	0.82	7.2	−11.2	−22.4	22.4
Acetylmethylcarbinol	0.35	3.1			6.4
Total			99.1	101.5	100.7

[1] VAN DER LEK, J. B., "Onderzoekingen over de Butylalkoholgisting," Delft, 1930.

TABLE 46.—FERMENTATION BALANCE OF GLUCOSE, USING *Cl. acetobutylicum*[1]
(Medium: yeast extract, 2 per cent glucose, 2 per cent calcium carbonate, 37°C.)

Products	Grams	Per cent of glucose fermented	Mols per 50 mols of glucose fermented		
			CO_2	H_2	Acetaldehyde
Glucose added	11.68				
Glucose unfermented	0.43				
Glucose fermented	11.25				
Carbon dioxide	5.34	47.5	97.0		
Hydrogen	0.2344	2.08	....	93.8	
Formic acid	Traces				
Acetic acid	1.15	10.2	....	−15.4	15.4
Butyric acid	2.97	26.6	....	......	61.8
Ethyl alcohol	0.39	3.5	....	6.8	6.8
Butyl alcohol	0.48	4.3	....	10.2	10.2
Acetone	Traces				
Acetylmethylcarbinol	0.28	2.5	....	......	5.2
Total	....	.....	97.0	95.4	99.4

[1] VAN DER LEK, J. B., "Onderzoekingen over de Butylalkoholgisting," Delft, 1930.

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CHAPTER XIII

THE ACETONE-ETHANOL FERMENTATION

The acetone-ethanol fermentation differs from the acetone-butanol fermentation in several respects, namely, the type of organism used; the end products, in particular with respect to the quantities of butyric acid and butanol formed; the optimum pH; the use of calcium carbonate; and the time required for the completion of the fermentation.

Schardinger[1] is credited with being the first to discover acetone as a product of bacterial fermentation. The organism isolated and investigated by him was designated as *Bacillus macerans*. Acetone, ethanol, acetic and formic acids were the principal end products obtained from the fermentation of potatoes or potato starch media containing peptone and calcium carbonate (see page 182).

At the suggestion of the Council of National Defense, Northrop undertook research in an attempt to develop acetone through a fermentation process. In 1919, Northrop, Ashe, and Senior[2] isolated an organism, *B. acetoethylicum* (*B. acetoethylicus* Northrop et al.),[3] which produced acetone from starch. A careful study was made of the organism and the biochemistry of the fermentation.

TABLE 47.—QUANTITIES OF ACETONE AND ALCOHOL PRODUCED BY *B. acetoethylicus* FROM VARIOUS SUBSTRATES[1]
(Medium: 2 per cent sugar; 0.5 per cent peptone, and 2 per cent $CaCO_3$; temperature, 37°C.; incubation period, 10 days)

Substance	Acetone, per cent	Alcohol, per cent	Substance	Acetone, per cent	Alcohol, per cent
Galactose	4– 5	22–24	Dextrin	6– 7	14–16
Maltose	6– 7	23–24	Dextrose	9–10	22–23
Mannose	6– 7	22–23	Levulose	8–10	24–25
Raffinose	8–10	22–23	Xylose	4– 5	18–20
d-Arabinose	6– 7	12–16	Glycerol	....	40–43
Calcium lactate	....	.....	Sucrose	8– 9	24–26
Starch	8–10	20–24			

[1] NORTHROP, J. H., L. H. ASHE, and J. K. SENIOR, *Jour. Biol. Chem.*, **39**: 1 (1919).

[1] SCHARDINGER, F., *Centr. Bakt. Parasitenk.*, Abt. II, **14**: 772 (1905).

[2] NORTHROP, J. H., L. H. ASHE, and J. K. SENIOR, *Jour. Biol. Chem.*, **39**: 1 (1919).

[3] "Bergey's Manual of Determinative Bacteriology," 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

B. acetoethylicus, which is closely related, if not identical, to *B. macerans*,[1] was isolated from some old potatoes. It is a motile, spore-forming, Gram-negative, facultative anaerobe. It grows well in a 2 per cent corn medium containing calcium carbonate. Its optimum reaction for growth is a pH of 8 to 9; for fermentation, a pH of 6 to 8. It has an optimum temperature of 40 to 43°C. Some of its spores will withstand boiling for at least 20 min. Ethyl, propyl, and butyl alcohols; acetone; and formic acid may be formed from suitable carbon-containing compounds. Table 47 shows some results of some fermentations.

Raw Materials.—A large number of carbohydrate substances may be used for the fermentation. Corn, potatoes, and molasses are substances available in large quantities and at a reasonable cost. Hydrolyzed corncobs and oat and peanut hulls may serve as cheap sources of raw material. It is sometimes necessary to add peptone to a mash to supply the nitrogen required by the organism, as, for example, when the mash contains starch alone. The use of concentrations of about 2 to 3 per cent of carbohydrate substance is customary.

pH.—According to Northrop and his associates,[2] the optimum pH range for the growth of *B. acetoethylicus* (*B. acetoethylicum*), was 8 to 9. However, highest yields were obtained when the fermentation mash was maintained at a pH of 6 to 8.

In the fermentation of xylose syrup, obtained from the hydrolysis of corncobs, Peterson and his associates[3] advised the use of an initial reaction of pH 7.6 to 8.4 and the provision of sufficient calcium carbonate to neutralize the acids as formed.

Arzberger, Peterson, and Fred[4] have shown that the reaction of the medium plays an important part in determining the relative quantities of the various end products of the fermentation by *B. acetoethylicus*. An increase in the pH of the mash resulted in a decrease of alcohol production and an increase in the volatile acid content. A marked acid reaction, a pH of 5.8 to 6.0, favored the production of acetone and decreased the yield of acids. These results are indicated in Fig. 29.

Calcium carbonate is always used in this type of fermentation to buffer the pH, in sharp contrast to the acetone-butanol fermentation. A 2 per cent concentration is satisfactory in most cases.

Optimum Temperature.—A temperature range of 40 to 43°C. is optimum for fermentations produced by *B. acetoethylicus*.

[1] *Ibid.*

[2] NORTHROP, J. H., L. H. ASHE, and R. R. MORGAN, *Jour. Ind. Eng. Chem.*, **11**: 723 (1919).

[3] PETERSON, W. H., E. B. FRED, and J. H. VERHULST, *Ind. Eng. Chem.*, **13**: 757 (1921).

[4] ARZBERGER, C. F., W. H. PETERSON, and E. B. FRED, *Jour. Biol. Chem.*, **44**: 465 (1920).

Duration of the Fermentation.—The fermentation ordinarily requires about 6 days. By using inert materials such as branches, coke, or corn-

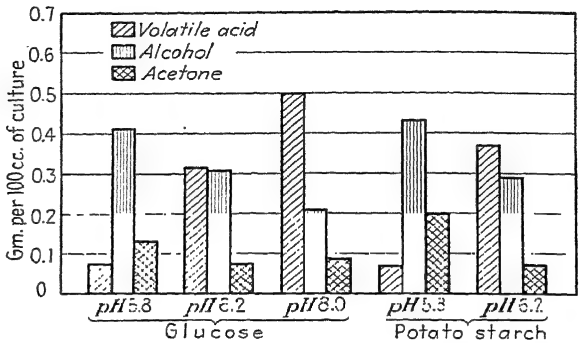

FIG. 29.—The influence of varying the reactions of the medium on the products of fermentation. [*Courtesy Arzberger, Peterson, and Fred, Jour. Biol. Chem.* **44**: 465 (1920).]

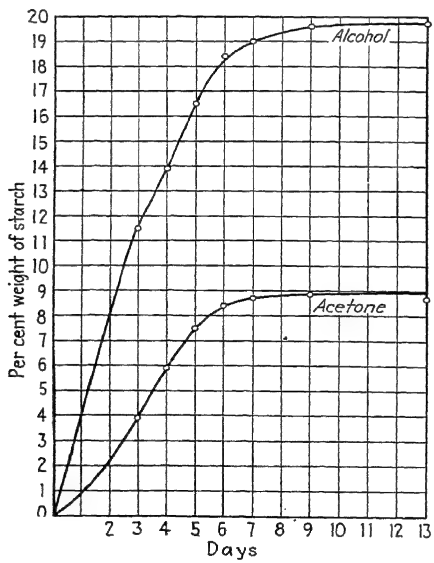

FIG. 30.—Rate of formation of acetone and alcohol. [*Courtesy Northrop, Ashe and Senior. Jour. Biol. Chem.* **39**: 1 (1919).]

cobs to retain the slime which is formed during the fermentation and which contains bacteria and calcium carbonate, the time required for

the fermentation may be considerably reduced. After the fermentation has been completed, the fermentation liquor is drawn off and a new mash added to the vat containing the slime-coated inert material. This process may be repeated several times, but it is necessary to use precautions to prevent contamination of the mash and to guard against sluggishness of the culture.

TABLE 48.—RATE OF FORMATION OF ACETONE AND ALCOHOL[1]
(Medium: 10 g. of potato starch, 4 g. of peptone, 10 g. of calcium carbonate, and 500 cc. of water; temperature, 42°C.; time analyzed as noted)

Time after inoculation, days	Culture No. 1		Culture No. 2		Culture No. 3		Average		Ratio alcohol to acetone	
	Acetone, per cent	Alcohol, per cent	Acetone, per cent	Alcohol, per cent	Acetone, per cent	Alcohol, per cent	Acetone, per cent	Alcohol, per cent	By weight	By mols
3	4.6	12.3	4.0	11.4	3.3	10.7	3.9	11.5	2.9	3.65
4	7.4	16.1	5.6	13.5	4.6	12.3	5.9	13.9	2.3	2.9
5	8.9	18.1	7.4	16.6	6.1	14.7	7.5	16.5	2.2	2.8
6	9.2	18.9	8.6	18.4	7.4	18.0	8.4	18.4	2.2	2.8
7	9.3	19.4	9.0	19.4	7.7	18.1	8.7	18.9	2.2	2.8
9	9.3	19.5	9.1	19.4	8.3	20.0	8.9	19.6	2.2	2.8
13	9.4	19.5	9.0	19.3	8.8	20.2	8.7	19.6	2.2	2.8

[1] NORTHROP, J. H., L. H. ASHE, and J. K. SENIOR, *Jour. Biol. Chem.*, **39**: 1 (1919).

The Fermentation of Corncobs.—Peterson, Fred, and Verhulst[1] (1921) devised a method for hydrolyzing corncobs and fermenting the sugars thus produced with *Bacillus acetoethylicus*. The corncobs were hydrolyzed for 1 hr. at a steam pressure of 20 lb. The ratio of the weights of the water, corncobs, and sulphuric acid used for the hydrolysis was 200:50:4. The hydrolyzate obtained was neutralized with calcium hydroxide and pressed, and the residue was washed. The sugar content of the mash was adjusted to a 3 per cent concentration (as glucose). The reaction of the fermentation was maintained between 7.6 and 8.4 at the beginning. Sufficient calcium carbonate was added to neutralize the acids formed. On the basis of 100 lb. of corncobs, the yield was: 2.7 lb. of acetone, 6.8 lb. of ethanol, and 3.4 lb. of volatile acids.

The Fermentation of Oat and Peanut Hulls.—In 1923, Fred, Peterson, and Anderson[2] reported that *B. acetoethylicus* (*B. acetoethylicum*)

[1] *Loc. cit.*

[2] FRED, E. B., W. H. PETERSON, and J. A. ANDERSON, *Jour. Ind. Eng. Chem.*, **15**: 126 (1923).

produced acetone and ethanol from the sugars obtained by the hydrolysis of oat and peanut hulls. (These hulls are waste products obtained in the manufacture of oatmeal and peanut butter.) The hulls were hydrolyzed under a pressure of 15 lb. of steam for 2 hr. with 2 per cent sulphuric acid. The hydrolyzate was neutralized with milk of lime, and the sugars were extracted by pressing and washing. From oat hulls, as high as 26.5 per cent of reducing sugars was obtained as glucose; from peanut hulls, 7.6 per cent. The sugar concentration of the mash was adjusted to approximately 3 per cent as glucose; calcium carbonate, peptone, and sodium phosphate were added, and the mash was inoculated with *B. acetoethylicus*. From 100 lb. of oat hulls, 7.2 lb. of ethanol, 3.9 lb. of acetone, and 1.4 lb. of volatile acids were produced.

For further details of this fermentation consult some of the references listed at the end of Chap. XII.

Mechanism of the Ethanol-acetone Fermentation.—Neuberg and his associates suggested that acetone was produced by either the amylobacter or macerans type of organism according to the following sequence:

$$\underset{\text{Acetaldehyde}}{2CH_3{\cdot}CHO} \rightarrow \underset{\text{Aldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO} \rightarrow \underset{\text{Acetoacetic acid}}{CH_3{\cdot}CO{\cdot}CH_2COOH} \rightarrow \underset{\text{Acetone}}{CH_3{\cdot}CO{\cdot}CH_3}$$

Speakman's Theory.—Speakman[1] contributed valuable additions to our knowledge of the biochemistry of the ethanol-acetone fermentation. He demonstrated that hydrogen gas, as well as carbon dioxide gas, was formed during the fermentation of carbohydrates by *B. acetoethylicus*. Previous workers had reported gas formation in terms of carbon dioxide only.

Using data derived from his own experiments and those of previous investigators, Speakman formulated a scheme to show the mechanism of the formation of the end products of the fermentation. In the normal fermentation of *B. acetoethylicus*, ethanol, but no acetone, is produced at the beginning of the fermentation; later both ethanol and acetone are formed simultaneously. Thus Speakman suggested that ethanol arose in two different ways: one involving a process in which no acetone was produced, the other associated with acetone formation.

Periodic neutralization of a fermenting mash with sodium hydroxide resulted in an increase in the volatile acid content and a decrease in the yields of ethanol and acetone. Hence, Speakman ruled out the possibility of a Cannizzaro reaction of the following nature:

$$\begin{array}{lcl} CH_3{\cdot}CHO + H_2 & & CH_3{\cdot}CH_2OH \\ \vdots & \rightarrow & + \\ CH_3{\cdot}CHO + O & & CH_3{\cdot}COOH \end{array}$$

Speakman demonstrated experimentally that pyruvic acid was produced by *B. acetoethylicus* from both glucose and maltose during the first

[1] Speakman, H. B., *Jour. Biol. Chem.*, **64**: 41 (1925).

half of the fermentations. Later acetone accumulated, but free pyruvic acid could not be detected. These facts suggested that pyruvic acid might be an intermediate in the formation of acetone.

In the fermentation of glycerol there was an almost complete absence of both pyruvic acid and acetone. Speakman reasoned that acetone was not produced because its precursor, pyruvic acid, was not formed, except possibly in traces, from glycerol. As an alternate explanation, he suggested that pyruvic acid, if produced in quantity, was converted to products other than acetone. He added pyruvic acid, neutralized with sodium hydroxide, to mashes containing glycerol. Acetone accumulated in the fermenting medium, part of it after the pyruvic acid had disappeared from the mash. Acetone was produced likewise from pure pyruvic acid. These facts indicated that pyruvic acid might be an intermediate product in the fermentation.

Since both formic and acetic acids were produced from pyruvic acid by fermentation with *B. acetoethylicus*, Speakman suggested that they were formed from pyruvic acid according to the following equation:

$$CH_3{\cdot}CO{\cdot}COOH + H_2O \rightarrow CH_3{\cdot}COOH + HCOOH$$

Lactic acid was produced rapidly in the fermentation mash during the initial stages (Speakman). The production then fell to a constant level and continued thus to the end of the fermentation.

Speakman proposed, as the result of the foregoing observations, the following scheme (1) for the production of substances during the first part of the fermentation:

1.

$$\begin{array}{ccccc}
 & & \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH} & & \\
 & & \uparrow + H_2 & & \\
\underset{\text{Glucose}}{C_6H_{12}O_6}\ (-2H_2) & \rightarrow & \underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + H_2 & \rightarrow & \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + CO_2 \\
 & & \downarrow + H_2O & & \downarrow + H_2 \\
 & & \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + \underset{\text{Formic acid}}{H{\cdot}COOH} & & \underset{\text{Ethanol}}{CH_3{\cdot}CH_2OH}
\end{array}$$

The following equations were suggested by Speakman to explain the changes that took place later in the fermentation, when acetaldehyde was found in the free state in the cell as the result of its more rapid formation than reduction:

2. $2CH_3{\cdot}CHO \rightarrow \underset{\text{Aldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO}$ (Condensation reaction)

$$\left\{\begin{array}{l} \underset{\text{Aldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO} \\ \\ \underset{\text{Acetaldehyde}}{CH_3CHO} \end{array}\right. \begin{array}{c} O \\ + \big| \\ H_2 \end{array} \rightarrow \begin{array}{l} \underset{\beta\text{-Hydroxybutyric acid}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}COOH} \\ + \\ \underset{\text{Ethanol}}{CH_3{\cdot}CH_2OH} \end{array} \quad \text{(Oxidation-reduction reactions)}$$

$$\left\{\begin{array}{l} \underset{\beta\text{-Hydroxybutyric acid}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}COOH} \\ \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} \end{array}\right. \xrightarrow{O} \begin{array}{l} \underset{\text{Acetoacetic acid}}{CH_3{\cdot}CO{\cdot}CH_2{\cdot}COOH} + H_2O \\ + \\ \underset{\text{Ethanol}}{CH_3{\cdot}CH_2OH} \end{array}$$

$$\underset{\text{Acetoacetic acid}}{CH_3{\cdot}CO{\cdot}CH_2COOH} \rightarrow \underset{\text{Acetone}}{CH_3{\cdot}CO{\cdot}CH_3} + \underset{\text{Carbon dioxide}}{CO_2} \quad \text{(Decarboxylation reaction)}$$

In the fermentation of glycerol there was more active hydrogen available than was the case in the fermentation of glucose. Thus acetaldehyde was reduced principally to ethanol in the fermentation of glycerol, while only a slight amount was condensed to aldol. An increased production of acetaldehyde followed the addition of pyruvic acid to the glycerol fermentation, but there was no proportional increase in the production of active hydrogen. Accordingly, some of the acetaldehyde condensed to aldol, and the second part of the Speakman scheme became operative.

Bakonyi's Theory.—Bakonyi[1] doubted the theory that acetone was produced from aldol through β-hydroxybutyric acid, for he stated that neither *B. macerans* nor *B. acetoethylicus* fermented β-hydroxybutyric acid readily.

In the fermentations carried on by the acetone-ethanol organisms, Bakonyi (1926) observed that ethyl alcohol and acetone were produced from carbohydrates, in the presence of calcium carbonate, in the proportion of 2 molecules of the former to 1 molecule of the latter. The addition of acetaldehyde or aldol to a fermenting mash resulted in an increase of ethanol and acetone in the proportion of 2 molecules of ethanol to 1 molecule of acetone. From 2 molecules of acetic acid (calcium acetate), 1 molecule of acetone was produced.

On the basis of the foregoing data, Bakonyi suggested that aldol was dismutated to ethyl alcohol and acetic acid, the acetic acid yielding acetone. (Compare with the butanol-acetone fermentation.)

The fermentation of starch would be thus expressed as follows:

$$\underset{\text{Starch}}{2C_6H_{10}O_5} + 3H_2O \rightarrow \underset{\text{Ethanol}}{2CH_3{\cdot}CH_2OH} + \underset{\text{Acetone}}{CH_3{\cdot}CO{\cdot}CH_3} + 5CO_2 + 4H_2$$

No matter what theory one accepts to explain the mechanism of the fermentation, it is necessary for the scheme selected to show balanced oxidation-reduction relationships and carbon partition. Complex changes take place in the breakdown of protein materials. It is important not to forget that here too changes are taking place which have a strong influence on the types and quantities of the end products of the fermentation.

References

See Chapter XII.

[1] Bakonyi, S., *Biochem. Zeit.*, **169**, 125 (1926).

CHAPTER XIV

THE BUTYL ALCOHOL–ISOPROPYL ALCOHOL FERMENTATION

The fermentation in which butyl alcohol and isopropyl alcohol are formed as the two principal products of commercial value is related in many respects to the acetone-butyl alcohol fermentation (Chap. XII) and the acetone-ethyl alcohol fermentation (Chap. XIII).

In fact, these three fermentations have many points of similarity both as to conditions that must be imposed for successful operation and as to the organisms involved. The bacteria causing these fermentations apparently all belong to the same group and are so nearly related that species differences are difficult to detect when they are described for classification purposes. In fermentations carried out on a sizable scale, however, the several species or strains show distinct differences in behavior and in the qualitative and quantitative analysis of the end products. Therefore it seems reasonable to regard them as separate fermentations.

Isopropyl alcohol (isopropanol) like butyl alcohol is a valuable solvent and can be used advantageously to replace ethyl alcohol or acetone in several industries, notably the manufacture of perfumes and toilet preparations.

The fermentation process may not become a very important one industrially since isopropyl alcohol is now obtained as a by-product in cracking petroleum in the manufacture of gasoline. The present demand is met in this way. As in the case of glycerol, the fermentation process may become economically advantageous under conditions of unusual demand.

Beijerinck[1] (1893) isolated an organism that produced butanol from malt sugar. This organism, although described as *Granulobacter butylicum,* has since been designated as *Clostridium butylicum* (Beijerinck) Donker.

The occurrence of isopropyl alcohol ($CH_3 \cdot CHOH \cdot CH_3$) as a fermentation product was observed by Pringsheim[2] in 1906. The organism that gave rise to isopropyl alcohol was named *Cl. americanum* Pringsheim.

[1] BEIJERINCK, M. W., *Verhandel. Akad. Wetenschappen Amsterdam, Afdeel. Naturkunde,* Qde Sectie II, No. 10 (1893).

[2] PRINGSHEIM, H. H., *Centr. Bakt. Parasitenk.,* Abt. II, **16**: 795 (1906).

This organism is now regarded as possibly identical with *Cl. butylicum* (Beijerinck) Donker.

Folpmers[1] (1920) isolated *Granulobacter butylicum* [*Cl. butylicum* (Beijerinck) Donker] from malt and demonstrated that the products of fermentation included not only butyl alcohol, but also isopropyl alcohol and small amounts of *n*-propyl and isobutyl alcohols, and acetic, butyric, and isobutyric acids.

Morikawa[2] isolated an organism designated as *Bacillus technicus* Prescott and Morikawa, which produced from *koji* mashes butanol; isopropyl alcohol; traces or small amounts of acetone, acetic, and butyric acids; and hydrogen and carbon dioxide. This organism appeared to differ in several respects from the organism described as *Cl. americanum* Pringsheim. The fermentation brought about by *B. technicus* has been studied by Morikawa and Prescott,[3] and by Dunn.[4]

Cl. butylicum (Beijerinck) Donker was studied by Van der Lek.[5]

Considerable research concerning various aspects of the butyl-isopropyl alcohol fermentation has been carried out by Osburn;[6] Osburn and Werkman;[7] Osburn, Brown, and Werkman;[8] Langlykke, Peterson, and McCoy;[9] Langlykke, Peterson, and Fred;[10] Sjolander, Langlykke, and Peterson;[11] and others.

Some General Considerations concerning the Fermentation.—There are several organisms that have the ability to produce isopropyl alcohol. These are closely related. In view of the fact that *Cl. butylicum* (Beijerinck) Donker is perhaps the best known of the isopropyl alcohol producers, this organism and the fermentations it produces will be considered in some detail.

Description of Cl. butylicum.[12]—*Cl. butylicum* is a sporeforming obligate anaerobe, possessing peritrichous flagella. It is Gram-positive in young

[1] Folpmers, T., *Tijdschr. Vergelijk. Geneeskunde*, Nos. 5–7 (1920–1922).

[2] Morikawa, K., "A New Butyl and Isopropyl Alcohol Fermentation," Thesis, Massachusetts Institute of Technology, Cambridge, 1926.

[3] Morikawa, K., and S. C. Prescott, *Jour. Bact.*, **13**: 58 (1927).

[4] Dunn, C. G., "A Biochemical Investigation of the Metabolic Activities of *Bacillus technicus*," Thesis, Massachusetts Institute of Technology, Cambridge, 1934.

[5] Van der Lek, J. B., Thesis, Delft, 1930.

[6] Osburn, O. L., *Iowa State Coll. Jour. Sci.*, **10**: 97 (1935).

[7] Osburn, O. L., and C. H. Werkman, *Ind. Eng. Chem.*, **27**: 416 (1935).

[8] Osburn, O. L., R. W. Brown, and C. H. Werkman, *Jour. Biol. Chem.*, **121**: 685 (1937); *Iowa State Coll. Jour. Sci.*, **12**: 275 (1938).

[9] Langlykke, A. F., W. H. Peterson, and E. McCoy, *Jour. Bact.*, **29**: 333 (1935).

[10] Langlykke, A. F., W. H. Peterson, and E. B. Fred, *Jour. Bact.*, **34**: 443 (1937).

[11] Sjolander, N. O., A. F. Langlykke, and W. H. Peterson, *Ind. Eng. Chem.*, **30**: 1251 (1938).

[12] Courtesy of Osburn, Brown, and Werkman, *Iowa State Coll. Jour. Sci.*, **12**: 275 (1938).

cultures but in old cultures may be Gram-negative. The vegetative cells of a 24-hr. culture grown at 37°C. in a 4 per cent corn mash containing potato extract are rod-shaped, 2 to 5 μ in length and 0.7 to 1.5 μ in width. These cells, which have rounded ends, occur singly, in pairs, or in chains. Granulose is present in young cells. Old cultures contain many spores.

The optimum temperature for growth is 37°C.

Cl. butylicum does not produce indol; reduce nitrates to nitrites; liquefy starch; assimilate peptone, unless carbohydrates are present; or utilize ammonium salts. It is catalase negative and forms hydrogen sulphide from sulphites, thiosulphites, and oatmeal.

Acid and gas are formed from amygdalin, arabinose, cellobiose. dextrin, dimethylglucoside, esculin, galactose, glucose, glycogen, inositol, inulin, lactose, levulose, maltose, melibiose, melezitose, raffinose, rhamnose, salicin, starch, sucrose, trehalose, and xylose. No acid or gas are formed from adonitol, dulcitol, erythritol, glycerol, mannitol, pectin, sodium lactate, or sorbitol.

The final products include butyl and isopropyl alcohols, carbon dioxide and hydrogen, small quantities of butyric and acetic acids, and possible traces of acetone and formic acid.

Granulobacter butylicum (Beijerinck) and *Cl. americanum* (Pringsheim) are names now considered to be synonyms of *Cl. butylicum* (Beijerinck) Donker.

Nitrogen Sources.—Some of the most favorable sources of nitrogen for the butyl-isopropyl alcohol organisms are yeast extract, peptone, malt sprouts, and corn steep. Each of these nitrogenous substrates contains protein in a partially hydrolyzed form. Other partially hydrolyzed proteins may be used as sources of nitrogen. *Cl. butylicum* does not hydrolyze proteins appreciably.

Malt sprouts have been used by Beijerinck, Folpmers, Osburn and Werkman, and others. One sample of malt sprouts used by Osburn and Werkman[1] contained 3.52 per cent of nitrogen, 10 per cent of maltose, and 18 per cent of starch and dextrin. Of the nitrogenous material, 35 per cent was soluble in hot water, and 30 per cent was in the form of amino nitrogen.

During the processing of corn in the manufacture of corn products, the clean corn is steeped for approximately 2 days in warm water containing a small quantity of sulphur dioxide. Soluble materials are extracted. The steep water is concentrated to approximately 12°Bé. and is known as "corn steep." In some of their work, Osburn and Werkman[1] used a sample of corn steep water containing 2.896 per cent of nitrogen. When the water was neutralized, 34 per cent of the nitrogenous substance

[1] OSBURN, and WERKMAN, *loc. cit.*

precipitated out. Of the total nitrogen left, 32 per cent was in the form of amino and amide nitrogen, and it was assumed that the remaining 34 per cent was contained in partially hydrolyzed protein and albumins.

Osburn and Werkman[1] demonstrated that from 5 to 11 per cent of isopropyl alcohol and from 19.5 to 25 per cent of butanol could be produced from 4 per cent glucose media containing mixtures of corn steep water and malt sprouts or corn gluten (the proteins of corn gluten are zein and glutelin principally).

Low yields of butanol (3 to 4 per cent) were obtained in the fermentation of 4 per cent corn mashes by *Cl. butylicum*, but the addition of 1 g. of yeast extract, or 5 cc. of corn steep water, per 300 cc. of mash resulted in vigorous fermentations that went to completion with the production of 12 to 14 per cent of butanol.[2]

The addition of asparagine,[3] yeast extract, or peptone to 5 per cent corn mashes caused large yields of butanol to be formed in place of butyric acid by *Cl. butylicum*. The stimulating effect of asparagine was proportional to the amounts used up to a concentration of 0.4 g. of asparagine per 300 cc. of 5 per cent corn mash.

Composition of Some Media.—In some of their research, Osburn and his associates[4] used a basal medium of the following composition:

	Per Cent
Glucose	2
Peptone	0.7
Difco yeast extract (powdered)	0.2
Dipotassium hydrogen phosphate	0.1

Langlykke and his coworkers[5] employed a medium containing the following ingredients:

	Per Cent
Glucose	3 (approx.)
Peptone	0.5
Asparagine	0.1
Dibasic ammonium phosphate	0.07

Neither peptone nor asparagine was satisfactory as the sole source of nitrogen.

Isopropyl Alcohol from Wood Sugars.—The utilization of wood sugars in the production of butyl and isopropyl alcohols has been studied by Sjolander, Langlykke, and Peterson.[6]

[1] Osburn, and Werkman, *loc. cit.*

[2] Osburn, and Werkman, *op. cit.*, **27**: 416–419 (1935).

[3] Brown, R. W., G. L. Stahly, and C. H. Werkman, *Iowa State Coll. Jour. Sci.*, **12**: 245–251 (1938).

[4] Osburn, O. L., R. W. Brown, and C. H. Werkman, *Jour. Biol. Chem.*, **121**: 685–695 (1937).

[5] Langlykke, Peterson, and Fred, *loc. cit.*

[6] Sjolander, Langlykke, and Peterson, *loc. cit.*

The wood hydrolyzates, similar to some obtained in the Scholler process (Chap. III), were treated to remove inhibitory substances. Excess calcium carbonate was added to neutralize the acidity of the hydrolyzates. The precipitate of calcium sulphate formed was removed by filtration, and the filtrate was adjusted to a pH of approximately 10 with lime to cause precipitation of iron and copper. Again the medium was filtered. The filtrate was acidified to a pH of about 6.5, clarified with norite, and adjusted to a pH of 6.0.[1]

As a result of experimentation, a nutrient medium containing the following constituents was used by Sjolander, Langlykke, and Peterson:

	Per Cent
Malt sprouts	2
Dried whole liver	0.25
Dibasic ammonium phosphate	0.05
Treated wood sugar solution	As indicated
Calcium carbonate	0.1

Calcium carbonate was sterilized separately and added, using aseptic precautions, to the sterilized medium. The inoculated mashes were incubated at 37°C. for 5 days under anaerobic conditions.

TABLE 49.—PRODUCTS OF FERMENTATIONS BY *Cl. butylicum*[1]

Carbohydrate source	Sugar as glucose		Neutral volatile products, per cent[2]	Distribution of neutral volatile products				Volatile acids,[3] milligrams per cubic centimeter	
	Original concentration, per cent	Fermented, per cent		Butyl alcohol, per cent	Ethyl alcohol, per cent	Acetone, per cent	Isopropyl alcohol, per cent	Acetic	Butyric
Hemlock 10	3.15	92	34.6	59	9	4	28	2.4	1.3
Beech 11	2.82	90	33.2	53	8	4	35	4.8	3.3
Glucose	2.90	99	29.8	70	7	3	20	1.0	1.1
Beech 11 (ether-extracted)	2.99	75	26.8	68	10	3	19	1.1	1.5
Glucose + acetate	2.95	98	28.7	49	1	9	41	4.3	2.7

[1] SJOLANDER, N. O., A. F. LANGLYKKE, and W. H. PETERSON, *Ind. Eng. Chem.*, **30**: 1251–1255 (1938).
[2] Based on the apparent sugar fermented.
[3] Volatile acids in uninoculated media (in milligrams per cubic centimeter):

	Acetic	Butyric
Hemlock 10	4.4	0
Beech 11	9.0	0.2
Beech 11 (ether-extracted)	0.4	0
Glucose + acetate	8.1	0.1

[1] *Ibid.*

The results of some experiments, using *Cl. butylicum*, are given in Table 49.

An examination of the foregoing table indicates that more isopropyl alcohol was formed from hemlock 10 and beech 11 wood sugars than from glucose. It was assumed that the higher yields of isopropyl alcohol from the media containing the wood hydrolyzates were due to the acetic acid contents of the uninoculated media. These contents were reduced during the fermentation.

In order to ascertain the correctness of the assumption that the increased yields of isopropyl alcohol were due to the acetic acid present in the uninoculated media, Sjolander and his associates carried out two experiments. In one experiment beech 11 wood hydrolyzate was extracted for 48 hr. continuously with ether. This ether-extracted medium was thus nearly freed of volatile acids. Table 49 shows that the products formed by fermentation of this medium were similar quantitatively to those produced from glucose. In the other experiment, a quantity of calcium acetate, equivalent to the acetic acid of the unextracted beech 11 wood hydrolyzate, was added to a medium containing glucose as the source of sugar. The percentage of isopropyl alcohol produced was over twice that produced in the glucose solution containing no calcium acetate, but the yield of butanol was considerably smaller.

TABLE 50.—FERMENTATION OF GLUCOSE BY *Cl. butylicum*[1]
(Medium: 2 per cent solution with 0.7 per cent peptone, 0.2 per cent yeast extract, and 0.1 per cent dipotassium phosphate)[1]

Experiment no.	Products per 100 millimols fermented							Carbon recovered, per cent	Oxidation-reduction index[2]
	Alcohols			Acids		Carbon dioxide, millimols	Hydrogen, millimols		
	Butyl, millimols	Isopropyl, millimols	Ethyl, millimols	Butyric, millimols	Acetic, millimols				
1	57.6	15.1	2.9	15.1	9.1	166.5	75.8	87.0	0.86
2	66.6	15.1	0	6.1	21.2	197.0	72.7	95.0	1.02
3	58.6	12.1	0	17.2	17.2	203.5	77.6	96.3	1.06
4	54.6	15.1	2.9	18.2	12.1	200.0	121.2	93.5	0.93
5	65.5	13.8	2.3	13.8	10.3	189.5	86.2	95.6	0.90
6	50.2	18.0	0	14.5	20.3	207.0	111.1	93.5	1.05
7	54.1	18.0	0	15.5	12.9	222.0	107.0	96.2	1.08

[1] OSBURN, O. L., R. W. BROWN, and C. H. WERKMAN, *Jour. Biol. Chem.*, **121**: 685 (1937).

[2] Ratio of oxidized to reduced products; a perfect ratio = 1.0 (cf. Erb, Wood, and Werkman, *Jour. Bact.*, **31**: 595 (1936), for method of calculation).

Yields of End Products.—The quantities of end products formed from glucose by *Cl. butylicum* in a series of laboratory experiments are shown in Table 50.

Effect of Neutralization with Sodium Bicarbonate.—Osburn and his associates[1] added sodium bicarbonate to fermenting glucose mashes with the result that butyl alcohol and isopropyl alcohol production was almost completely suppressed, the formation of isopropyl alcohol being inhibited more markedly than that of butanol. In the presence of relatively large concentrations of sodium bicarbonate, salts of acetic, butyric, formic, lactic, and pyruvic acids accumulated in the mash.

In carrying out the experiments, Osburn, Brown, and Werkman added an 8 per cent solution of sodium bicarbonate to the inoculated glucose mashes after the fermentations had become vigorous, usually after about 14 hr. In each case, the solution was added rapidly at first and then gradually until the weight of sodium bicarbonate added became equal to the weight of the sugar being fermented. The results of these experiments are shown in Table 51.

TABLE 51.—FERMENTATION OF 2.0 PER CENT GLUCOSE BY *Cl. butylicum*
(In presence of sodium bicarbonate, 0.7 per cent peptone, 0.2 per cent yeast extract, 0.1 per cent dipotassium phosphate)[1]

Experiment no.	Products calculated per 100 millimols glucose fermented							Carbon dioxide, millimols	Hydrogen, millimols	Carbon recovered, per cent	Oxidation-reduction index	pH
	Alcohols		Acids									
	Butyl, millimols	Isopropyl, millimols	Butyric, millimols	Acetic, millimols	Pyruvic, millimols	Lactic, millimols	Formic, millimols					
1	3.0	0	54.0	24.0	3.0	43.5	0	135.0	135.9	91.7	1.06	7.2
2	3.0	3.0	51.0	33.0	12.0	37.8	0	138.0	150.0	96.5	1.05	7.1
3	5.4	1.8	33.7	27.0	11.2	56.2	27.0	91.5	87.4	89.4	1.21	6.7
4	6.7	1.9	30.6	35.4	7.6	60.2	47.8	74.6	57.9	92.0	1.35	6.9

[1] OSBURN, O. L., R. W. BROWN, and C. H. WERKMAN, *Jour. Biol. Chem.*, **121**: 685 (1937).

Methylglyoxal was isolated from the fermenting mashes and identified.

Isopropyl Alcohol Production by Bacillus technicus.—An organism isolated from *koji* rice and described in 1926 by Morikawa[2] produced butyl and isopropyl alcohols, acetic and butyric acids, carbon dioxide and hydrogen and traces or small amounts of acetone as the principal end products from suitable nutrient carbohydrate media. Hydrolyzed

[1] OSBURN, O. L., R. W. BROWN, and C. H. WERKMAN, *Jour. Biol. Chem.*, **121**: 685 (1937).

[2] MORIKAWA, *loc. cit.*; PRESCOTT, S. C., and K. MORIKAWA, U.S. Patent 1,933,693, Nov. 7, 1933.

koji rice; malt sirup; cerelose; mixtures of dextrins, maltose and glucose; or mixtures of hydrolyzed *koji* rice with certain other sugars were readily fermented. Unhydrolyzed corn mashes, purified glucose or maltose, and blackstrap molasses were poorly fermented with low yields. The optimum concentration of sugar was 10 to 13 per cent; the optimum reaction, pH 5 to 7. Good yields were obtained at temperatures of 28 to 37°C. Calcium carbonate stimulated the formation of neutral solvents.

Isopropyl Alcohol Production by Butyric Acid Anaerobes.—Some butyric acid anaerobes produce isopropyl alcohol. However, the principal neutral solvent formed is butyl alcohol. Ethyl alcohol is formed in small quantities, while variable amounts of acetone are produced. In the following table are given some data concerning the results of fermentations of glucose by butyric acid anaerobes.

TABLE 52.—FERMENTATIONS CHARACTERIZED BY THE PRODUCTION OF ISOPROPYL ALCOHOL[1]

Culture no.	Glucose fermented,[2] per cent	Acidity 0.1 N NaOH in 10 cc., cc.	Neutral volatile products based on glucose fermented				
			Butyl alcohol, per cent	Ethyl alcohol, per cent	Isopropyl alcohol, per cent	Acetone, per cent	Total, per cent
21	94.9	3.95	19.2	2.5	4.7	0.5	26.9
22	94.9	3.70	22.2	2.2	4.4	0.7	29.5
36	94.1	3.75	19.3	2.3	5.2	0.9	27.7
18	78.0	5.30	17.8	2.1	4.5	0.6	25.0
24	75.1	4.85	16.6	2.8	6.1	0.8	26.3
20	94.8	4.30	15.7	2.4	3.2	1.6	22.9
30	93.1	3.65	21.0	1.7	3.8	1.3	27.8
46	94.5	3.30	17.1	2.4	3.8	3.7	27.0

[1] LANGLYKKE, A. F., W. H. PETERSON, and E. MCCOY, *Jour. Bact.*, **29**: 333–347 (1935).
[2] The medium consisted of a double-strength yeast water ("the clear water extract of fresh, starch-free yeast, 200 g. per liter of tap water") containing 2.5 per cent glucose with the reaction adjusted to pH 7.0.

Effect of Adding Products to Fermenting Glucose Media.—The effect of adding various substances to glucose mashes being fermented by *Cl. butylicum* has been studied recently by Langlykke and Fred; by Langlykke, Peterson, and Fred; and by Osburn, Brown, and Werkman.[1]

[1] LANGLYKKE, A. F., and E. B. FRED, *Jour. Bact.*, **33**: 102 (1937).
LANGLYKKE, PETERSON, and FRED, *op. cit.*, **34**: 443–453 (1937).
OSBURN, O. L., R. W. BROWN, and C. H. WERKMAN, *Iowa State Coll. Jour. Sci.*, **12**: 275–284 (1938).

In a normal fermentation of glucose by *Cl. butylicum*, acetone accumulated in the fermenting medium before the formation of isopropyl alcohol was observed.[1] Acetone, a hydrogen acceptor, when added to a fermenting glucose medium was converted to isopropyl alcohol. Acetic acid, when added, was transformed to isopropyl alcohol. Acetaldehyde was converted to ethanol, butyric acid to butanol, and acetylmethylcarbinol to 2:3-butyleneglycol under similar conditions. Pyruvic acid increased the yields of the products normally formed from glucose. Calcium or sodium pyruvate yielded acetic and butyric acids. Lactic acid was not fermented by *Cl. butylicum*.

Acetone and other hydrogen acceptors caused increased yields of isopropyl alcohol and acetone from glucose but decreased the yields of butyric acid and butanol. Since the added hydrogen acceptors utilized hydrogen, less was available for hydrogenation reactions.[1]

Mechanism for the Formation of Isopropyl Alcohol.—A scheme described by Kluyver, based on the proposals of Donker and Van der Lek, to explain the intermediary reactions involved in the formation of butanol, ethanol, isopropyl alcohol, acetone, and other end products has been presented in Chap. XII. According to this scheme, acetone and isopropyl alcohol were formed from acetic acid through the intermediary substance, acetoacetic acid.

References on the Butyl Alcohol–Isopropyl Alcohol Fermentation

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CHAPTER XV

THE ACETIC ACID BACTERIA AND SOME OF THEIR BIOCHEMICAL ACTIVITIES

The acetic acid bacteria belong to the family *Acetobacteriaceae*.[1] The cells are rod-shaped, but elongated, filamentous, club-shaped, swollen, or branched forms may occur. They may be motile or nonmotile and do not form endospores. The bacteria may secure energy by the oxidation of ethanol to acetic acid, by the oxidation of various sugars and other alcohols, or by anaerobic dissimilations.

The following members of the genus *Acetobacter* are listed and described in "Bergey's Manual":[1]

Acetobacter aceti (Kützing) Beijerinck (the type species)
A. pasteurianum (Hansen) Beijerinck
A. kuetzingianum (Hansen) Bergey et al.
A. zeidleri Beijerinck
A. acetosum (Henneberg) Bergey et al.
A. xylinum (Brown) Bergey et al.
A. hoshigaki (Takahashi and Asai) Bergey et al.
A. ascendens (Henneberg) Bergey et al.
A. plicatum Fuhrmann
A. acetigenum (Henneberg) Bergey et al.
A. oxydans (Henneberg) Bergey et al.
A. industrium (Henneberg) Bergey et al.
A. rancens Beijerinck
A. melanogenum Beijerinck
A. suboxydans Kluyver and de Leeuw

Henneberg has described the five following species of acetic acid bacteria, which are listed in "Bergey's Manual":

Bacterium schuetzenbachii Henneberg
Bact. xylinoides Henneberg
Bact. orleanense Henneberg
Bact. vini acetati Henneberg
Bact. curvum Henneberg

In addition, brief descriptions of *A. viscosum* Shimwell and *A. capsulatum* Shimwell appear in the "Manual."

Biochemical Activities of the Acetobacter.—The biochemical activities of the *Acetobacter* consist mainly of aerobic and anaerobic dissimilations

[1] "Bergey's Manual of Determinative Bacteriology," 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

and the synthesis of polysaccharides. The aerobic dissimilations are, from the industrial viewpoint, most important, including the oxidative dissimilation of sugars and alcohols. The oldest and best known of the fermentations brought about by acetic acid bacteria is that in which acetic acid or vinegar is produced.

VINEGAR

Vinegar may be defined as the condiment made from sugary or starchy materials by alcoholic and subsequent acetous fermentations. The term literally signifies "sour wine," according to its derivation from the French (*vinaigre* = *vin*, "wine," plus *aigre*, "sour" or "sharp").

Composition.—The composition of a vinegar will depend somewhat on the nature of the raw material that has undergone alcoholic and acetous fermentations. The conditions of manufacture, aging, and storage will also influence the composition of the product. In a cider vinegar, for example, one might find, besides at least 4 g. of acetic acid (CH_3-COOH) per 100 cc. of vinegar at 20°C., traces or small amounts of alcohol, glycerin, esters, reducing sugars (as invert sugar), pentosans, salts, and other substances.[1]

The Food and Drug Administration of the United States[2] has adopted the following definitions and standards for vinegars:

Vinegar, cider vinegar, apple vinegar. The product made by the alcoholic and subsequent acetous fermentations of the juice of apples. It contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

Wine vinegar, grape vinegar. The product made by the alcoholic and subsequent acetous fermentations of the juice of grapes. It contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

Malt vinegar. The product made by the alcoholic and subsequent acetous fermentations, without distillation, of an infusion of barley malt or cereals whose starch has been converted by malt. It contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

Sugar vinegar. The product made by the alcoholic and subsequent acetous fermentations of sugar sirup, molasses, or refiners sirup. It contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

Glucose vinegar. The product made by the alcoholic and subsequent acetous fermentations of a solution of glucose, is dextrorotatory and contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

Spirit vinegar, distilled, vinegar, grain vinegar. The product made by the acetous fermentation of dilute distilled alcohol. It contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

[1] BROOKS, R. O., "Critical Studies in the Legal Chemistry of Foods," Reinhold Publishing Corporation, 1927.

[2] U.S. Dept. of Agriculture, F.D.A., Service and Regulatory Announcements, Food and Drug, No. 2, Rev. 5, November, 1936.

Historical.—Although vinegar has been known for thousands of years, its microbiological nature was not realized until a little more than 100 years ago, when Kützing (1837) reported that the conversion of ethanol to acetic acid was brought about by living microorganisms. Fifteen years earlier, Persoon had given the name *Mycoderma* to the film that formed on liquids in which an acetic fermentation was taking place.

It remained for Pasteur (1868) to confirm Kützing's opinion and to prove the physiological nature of the acetic acid fermentation. Pasteur, however, believed that the fermentation was caused by a single species of bacteria, *Mycoderma aceti*. In 1878, Hansen showed that more than one species of bacteria could bring about the souring of beer, *i.e.*, the oxidation of the ethanol to acetic acid. He isolated and named *Bacterium aceti* and *Bact. pasteurianum*. At a later date he isolated *Bact. kützingianum*, while a fourth species was described by Brown. About 1897 Henneberg studied and reclassified the group and described several other species.

Nomenclature.—The literature contains occasionally more than one name for the same species of *Acetobacter*. For example, *A. aceti* has been referred to both as *M. aceti* and *Bact. aceti*. The system of nomenclature adopted in "Bergey's Manual" will be used in this chapter.

General Requirements for Manufacture.—In the manufacture of vinegar several factors are worthy of special consideration: the selection of the microorganism; the nature of the raw material; the concentration of the ethanol used, as well as that of the vinegar added at the start to acidify it; the amount of oxygen supplied; the nature of the supporting medium; the temperature of the fermentation; aging and storage; clarification; bottling and pasteurization; and the character and composition of the tanks, containers, and fixtures coming in contact with the vinegar during the manufacturing process.

Selection of Microorganism.—Although there are a large number of bacteria, as well as other microorganisms, that have the ability to produce acetic acid in small amounts from various substrates, only relatively few bacteria possess the characteristics desired for vinegar production. *Bact. schue zenbachii* or *Bact. curvum* may be used to produce acetic acid from ethyl alcohol in the quick vinegar process, while *Bact. orleanense* may be used in either the quick vinegar or Orleans process. *Acetobacter aceti*, *A. pasteurianum*, *A. xylinum*, *A. ascendens*, and *A. acetigenum* may be isolated from vinegar.

Nature of Raw Material.—Vinegar may be manufactured from almost any product capable of yielding alcohol by fermentation. Fruits, such as apples, grapes, pears, peaches, plums, figs, and oranges; berries; honey; sugar-containing sirups; hydrolyzed starchy materials; beer; and wine may serve as raw materials for vinegar manufacture.

Wine and apple juice, or cider, are two of the best raw materials for vinegar production—wine being used to a large extent in France, Italy, Spain, and Greece; cider in the United States. Vinegar prepared from malt is popular, especially in England, while that from honey is considered to be very palatable.

It is obvious that the quality of the vinegar will depend in large part on the quality of the raw material used. Fruit should be clean, sound, and in the proper state of maturity; wine or alcoholic media should be clear, clean, and free from preservatives. Cleanliness of the plant, equipment, and surroundings are likewise indispensable for the production of a high-grade product.

Yeast Fermentation.—Before the acetic acid fermentation can take place, the sugar in the fruit juice or other sugar-containing medium must be converted to alcohol by yeast fermentation. The yeast naturally present in the fruit juice may bring about a successful spontaneous fermentation, but the manufacturer cannot rely on chance and should use a starter in order to ensure a suitable fermentation. Although compressed yeast may be often used satisfactorily as a starter, the use of a selected wine yeast, for example, *Saccharomyces ellipsoideus*, generally improves the flavor of the final product. Fermentations may be carried out favorably at 75 to 80°F. (23.9 to 26.7°C.).[1] It is advisable to follow the course of the fermentation with hydrometers (Brix or Balling) that indicate the approximate percentage of sugar present in the fermenting mash. When the fermentation is complete, yeast, pulp, and other sediment should be removed from the medium by a process of settling. A storage period of 2 to 3 weeks is usually allowed for the sedimentation, after which the clear medium is "racked" (drawn off), adjusted, if necessary, to the optimum alcohol concentration, and acidified by the addition of some pure vinegar.

Concentration of Alcohol.—Adjustment of the alcohol content of the medium may be necessary in order to ensure a successful fermentation. Alcohol in a concentration of 10 to 13 per cent is readily fermented. When using alcohol concentrations of 14 per cent or greater, the zoogloeal mat forms with difficulty and the alcohol is incompletely oxidized to acetic acid. On the other hand, the use of too low concentrations may result in the loss of vinegar, for, when the concentration of ethanol is less than 1 or 2 per cent, esters and acetic acid are oxidized with the loss of aroma and flavor. Carbon dioxide and water are formed from acetic acid:

$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O$$

With some species of acetic bacteria this action may occur even in normal concentrations.

[1] Cruess, W. V., and M. A. Joslyn, *Calif. Agr. Expt. Sta., Circ.* 332, 1934.

Acidification.—The initial acidification is carried out with two objects in view: to inhibit the development of undesirable types of bacteria and to supply desirable acetic-acid-producing bacteria for seed purposes. The amount of vinegar added to the alcoholic medium will depend upon the nature of the process, but usually 10 to 25 per cent by volume of strong vinegar is considered to be sufficient. If the mix is to be permitted only one passage through a generator, the initial acidity may be adjusted to 3 to 3.5 per cent and sufficient alcoholic substrate added to yield a vinegar with a final acid strength of approximately 6 per cent.[1]

A medium should never be acidified before the alcoholic fermentation is complete, because the sugar in the medium would be incompletely converted to alcohol after the addition of acetic acid. Vinegars made from incompletely fermented juices are usually low in acetic acid and of poor quality.

Oxygen.—Since the conversion of ethanol to acetic acid is primarily an oxidation process, or a dehydrogenation in which atmospheric oxygen acts as the hydrogen acceptor, the success of the fermentation will depend in no small part on the availability of large quantities of oxygen. The following equations will illustrate the requirement for oxygen:

$$\underset{\text{Ethanol}}{CH_3CH_2OH} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Acetaldehyde}}{CH_3CHO} + H_2O$$
$$\underset{\text{Acetaldehyde}}{CH_3CHO} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Acetic acid}}{CH_3COOH}$$

If fermentation is to go on in casks or barrels they should be provided with an adequate number of holes above the surface of the liquid to ensure a plentiful oxygen supply. The openings should be screened to keep out insects, and the barrels should be only partly filled. When generators are used they should be packed rather loosely with zoogloea-supporting materials to permit sufficient ventilation in all parts of the generator.

Commercial production of vinegar and acetic acid by fermentation is usually carried out in large casks of proper design or in generators constructed in the form of a truncated cone provided at top and bottom with perforated scaffolds, and near the bottom with air inlets. The generator thus acts as a stack or chimney in which a strong upward current of air takes place, bringing the oxygen in contact with the bacterial film resident on the surfaces of the supporting material for the film. (In the section on methods of manufacture, the construction will be taken up in greater detail.)

The Supporting Medium.—The use of a satisfactory supporting medium has much to do with the duration and success of the acetic fermentation, since its purpose is to increase greatly the area or surface

[1] Cruess, W. V., "Commercial Fruit and Vegetable Products," 2d ed., McGraw-Hill Book Company, Inc., New York, 1938.

exposed and thus accelerate the fermentation by virtue of the availability of a larger oxygen supply.

Many manufacturers of vinegar during and before the time of Pasteur failed to realize the necessity for an adequate oxygen supply and the use of a support for the film in containers where the zoogloeal mat was likely to be disturbed periodically. Disturbed films sank and used up the nutrient materials anaerobically without producing acetic acid. Pasteur pointed out defects in the processes and made suggestions as to how they could be improved.

A "raft" or light wooden grating may be used as a support for the bacterial film in the cask or barrel. In the rapid fermentation process, where generators are used, the supporting medium is usually constructed of beechwood shavings or chips, although rattan, wood charcoal, coke, pressed pomace, corncobs, excelsior, or other materials that offer large surface areas may be substituted. Coke is more durable than wood charcoal, while corncobs are not particularly durable.

It is essential that the material used for supporting purposes should impart no undesirable odors or flavors to the vinegar. The material should be thoroughly extracted with water and then with vinegar before it is used in the generator.

Temperature Relations of the Acetic Acid Bacteria.—The acetic group of bacteria is characterized by very definite and peculiar temperature relations. At temperatures below 12 to 15°C. it grows slowly, and the cells are short but unusually broad. From 15 to 34°C. they appear to develop in what may be called the "normal" manner, growing rapidly and developing chains of cells of varying number of units or elements. In suitable media the walls become swollen and exhibit the early stages of zoogloea formation. At still higher temperatures (approximately 42 to 45°C.) long thread-like transparent filaments with no cross walls and with irregular bulging and occasional branching have been observed. This condition appears to be a pathological state induced by high temperature, and if the culture is long maintained under these conditions it may lose its power to function normally. A prompt return to temperatures of 15 to 34°C. will, however, result in the production of some cells of normal appearance and behavior.

This strange temperature effect is of importance in determining the most advantageous thermal conditions for active fermentation in vinegar manufacture.

The exact temperature to be used will depend on the organism and process being employed. In the United States a temperature of 80 to 85°F. (26.7 to 29.4°C.) is usually favorable for the acetic acid fermentation. In England, a higher temperature is sometimes used. The use of too low a temperature favors a slow fermentation, while the use of too

high a temperature favors the loss through evaporation of alcohol, acetic acid, and the volatile substances important in the production of flavor and aroma.

Storage.—Previous to storage, the vinegar fermentation should be allowed to proceed until the vinegar has reached its maximum strength. After this stage in the fermentation, when all the alcohol has been converted to acetic acid, the acetic acid bacteria or their enzymes will gradually destroy the vinegar by oxidation, unless they are inhibited through exclusion of oxygen or other means. Barrels or tanks, therefore, in which vinegar is to be stored, should be completely filled and then sealed to prevent access of air to the vinegar.

Aging.—Aging is a process that improves the flavor and clarity of vinegar, especially vinegars made from wine or fruit juices. Esters are formed and the harsh flavor and odor of the fresh product disappear (see Chap. VI). Aging takes place during storage and may require a year or longer. Alcohol vinegars, which are essentially dilute acetic acid solutions, are not improved by aging.

Clarification.—Some vinegars may be bottled without further treatment, but most of them should be clarified first. Clarification may be effected by filtration, using filter aids, or by fining. The former method is to be preferred, however.

In the process of fining, a substance such as fish isinglass or bentonite or Spanish clay is thoroughly mixed with the vinegar, the mixture is permitted to stand until a clear vinegar appears, and finally the clarified vinegar is drawn or siphoned off. Usually 1 oz. of fish isinglass, ½ lb. of bentonite or 1 to 5 lb. of Spanish clay will be sufficient to clarify 100 gal. of vinegar.[1]

Bottling and Pasteurizing.—The vinegar should be properly aged, clear, and bright before it is bottled. Bottles should be completely filled and tightly capped or corked with treated corks to prevent access of air. Pasteurization is carried out by heating the bottles to a temperature of 60 to 66°C. (140 to 150°F.) for about 30 min., or until the temperature of the vinegar within the bottles has reached at least 60°C. (140°F.). An alternative method is to pasteurize the vinegar in bulk, cool it to 21.1°C. (70°F.), and then bottle it.[2]

Treatment of vinegar with silver ions by passing the vinegar between silver electrodes, through which an electric current is passing, has been practiced to some extent during the past few years. The silver tends to prevent development of the bacteria present in the vinegar by acting as a germicide and antiseptic.

[1] Cruess, W. V., and M. A. Joslyn, *Calif. Agr. Expt. Sta., Circ.* **332**, 1934.
[2] *Ibid.*

Composition of Containers and Equipment.—Since vinegar is corrosive, contact with certain metals should be avoided in its manufacture, storage, and distribution. Iron is readily attacked and eventually dissolved and may cause a clouding and browning of the vinegar (see the section on the darkening of vinegar). Zinc may produce an undesirable flavor in the product and, in addition, form a poisonous zinc acetate. Galvanized iron equipment is therefore unsatisfactory on account of the zinc contained in it. Copper and brass should not be used as they may likewise be attacked and affect the flavor of vinegar adversely.

Wood, aluminum, glass, hard rubber, and pressed paper may be used satisfactorily in contact with vinegar.

Methods of Manufacture.—Vinegar is made as a home industry by simply providing an air supply and allowing barrels of cider or wine to ferment spontaneously. This does not always produce a high-grade product.

The commercial methods of manufacture include slow and rapid processes. A few typical illustrations of methods will follow:

Orleans Process.—Of the slow processes, the Orleans, or "French method," is the oldest and also the best for the production of table vinegars. Barrels of approximately 200-liter (52.8-gal.) capacity are used as the containers in this process. Each barrel is filled about one-third full with a good grade of vinegar, which constitutes the starter or culture, and 10 to 15 liters of wine are added. At weekly intervals for 4 weeks, the same amount of wine is added to the barrel. When 5 weeks have passed, 10 to 15 liters of vinegar are withdrawn from the barrel, which is now about one-half filled, and the same amount of wine is introduced. The operation may be repeated, thus becoming a slowly continuous process.

Air is admitted to the barrels through holes—one usually at each end of the barrel, which is placed on its side—above the level of the vinegar medium. These holes are at least 1 in. in diameter and screened to prevent the entrance of insects. Air may also be admitted through a top bunghole likewise screened. The acetic acid bacteria form a thin film on the surface of the solution, and this film later becomes quite thick and gelatinous. This gelatinous zoogloeal mat, which contains very large numbers of bacteria, is known as the "mother of vinegar." Eventually, unless supported on a "raft" or framework, it will sink to the bottom of the barrel and a new film will form.

Although vinegar of high grade is produced by this method, it is a slow and costly process that involves much attention. The films are easily disturbed by the addition of the alcoholic medium and the withdrawal of vinegar. If they sink, they use up the nutrient substances but fail to produce acetic acid under anaerobic conditions. Pasteur made

suggestions as to how to improve this process. One such suggestion involved the use of a support for the film.

Modifications of the Orleans Process.—Most of the slow methods are modifications of the old Orleans process. A light grating of wood may be floated on the liquid medium to support the bacterial film and prevent it from breaking up and sinking. Another method[1] to prevent submergence of the film is to equip the top bunghole with a funnel attached to a glass tubing that leads to the bottom of the barrel. Alcoholic solutions can

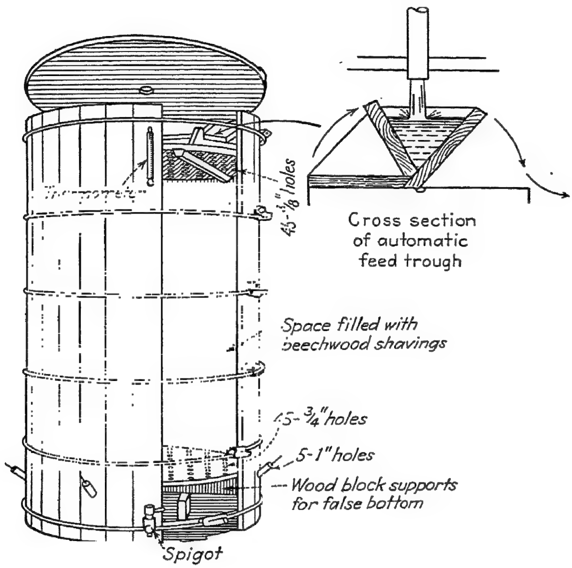


FIG. 31.—Generator for quick process. (*Courtesy E. LeFevre, U.S. Dept. Agr., Farmers' Bull.* 1424, 1936.)

be added with a minimum disturbing effect on the film. A glass tube may be attached through a bunghole in the bottom of the barrel to serve as a gauge for measuring the level of the liquid in the barrel during fermentation and as a means for withdrawing finished vinegar without disturbing the zoogloeal mat.

The Quick Vinegar Process.—The quick vinegar process, now largely used, is also known as the "German process." Boerhave discovered in the early part of the nineteenth century that when wine was permitted to trickle down through a tall receptacle containing loosely packed pomace, vinegar was rapidly produced. Schutzenbach (1823) modified the method of Boerhave by introducing other types of porous material in

[1] *Ibid.*

order to obtain maximum contact of the organisms with air. The method used by Schutzenbach is the basis for modern methods of manufacture using the generator.

Generators.—Generators are of various sizes and shapes. Some are 10 ft. in diameter and 20 ft. high. Some are 8 by 16 ft. or 4 by 8 ft.[1] The generator is equipped with a false perforated bottom, through which air enters. Some of the larger generators have a perforated shelf approximately halfway between the top and the bottom of the tank, which aids in supporting the beechwood shavings or other material used to present a large surface area for the acetic acid bacteria. The use of the perforated shelf prevents crushing and matting of the shavings due to the weight of the superimposed material. Near the top of the generator above the shavings there is a false top or perforated plate over which is arranged a rotating sprinkler, or sparger, for producing a uniform distribution of the vinegar stock (vinegar plus alcohol-containing substrate) over the top surface of the supporting material. In place of a sparger at the very top of the generator there may be located a tilting trough or other automatic device, which periodically dumps vinegar stock upon the distributing ad.

The vinegar stock may be passed through the same generator until the desired acidity is obtained, or it may be passed through two or three sets of generators connected in series, each with increasing acidity. The latter method of operation is known as "tandem operation."

A generator 10 ft. in diameter and 20 ft. high usually produces 80 to 100 gal. of distilled vinegar per day.[2]

The Frings Method.—Vinegar may be manufactured by the Frings process.[3] This process, in fundamentals, is similar to the quick generator process, but it possesses several advantages, which will be mentioned later.

FRINGS GENERATOR.—Figure 32 shows a cross section of the Frings generator. The generator consists of an airtight tank, which is usually 14 ft. in diameter and 15 ft. in height, and certain accessory equipment. The tank is superimposed on concrete beams, in such a manner that air may circulate beneath the apparatus. Inside the tank, near the bottom, is a wooden grating, which supports beechwood shavings. These shavings are piled to within about 1.5 ft. of the top of the generator. Below the wooden grating at the bottom of the tank is the collection chamber of the generator. At the very bottom of this chamber is a cock or faucet through which the finished product is withdrawn. Near the bottom of

[1] FETZER, W. R., *Food Industries*, **2**: 489 (1930).

[2] *Ibid.*

[3] HANSEN, A. E., *Food Industries*, **7**: 277 (1935); FRINGS, H., U.S. Patent 1,880,381, Oct. 4, 1932.

the tank, cooling coils are also located. The vinegar mix, which has passed down through the shavings, is circulated through an inner pipe made of stainless steel, or other acid-resistant substance, and is cooled by the passage of water through a surrounding outer pipe of copper. The mix is then forced by means of a centrifugal pump made of stainless steel from the cooling coils to the top of the tank through a rubber line. The

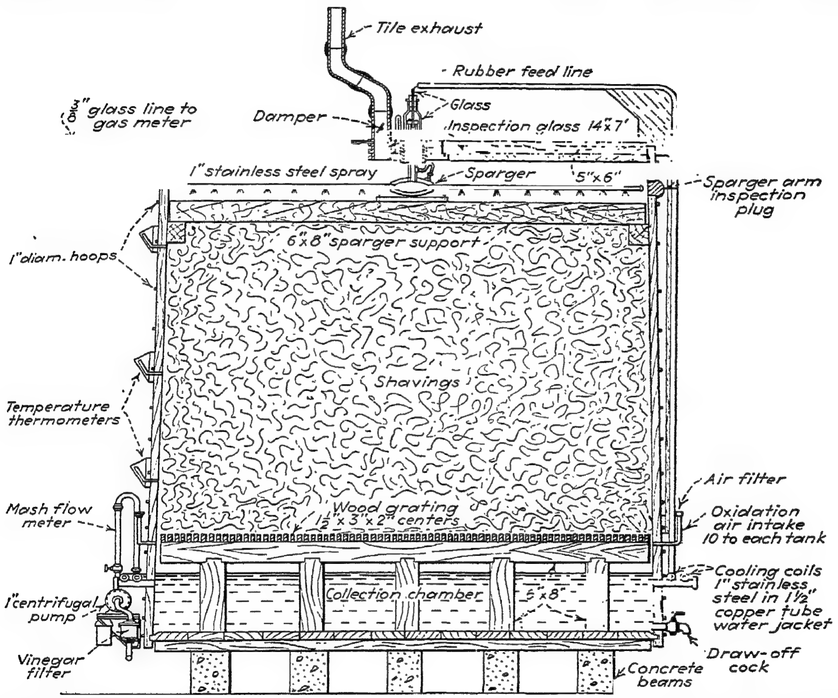


FIG. 32.—Cross section of the Frings generator. [*Courtesy A. E. Hansen, Food Industries*, **7**: 277 (1935).]

mix passes from the rubber feed line through a glass connecting device to a sparger, or distributing arm, constructed of stainless steel.

The oxygen supply of the generator is controlled by a damper, located in a 4-in. vent constructed at the top of the tank. This vent constitutes the air outlet of the generator. Air enters the generator through a series of intakes (10) located around the tank near the level of the wooden grate. These inlets contain air filters.

Thermometers are located at different points in the generator in order to determine the temperatures in the different parts.

The generator contains a meter, attached near the pump, for measuring the rate of flow of the mix or medium, also a meter for recording the rate of flow of the cooling water.

A generator of the type just described may produce approximately thirty times the quantity of white vinegar that can be produced by a tank (4 by 8 ft.) of the noncirculating type.[1]

COMPOSITION OF THE MIX.—A 2,500-gal. mix may contain 10.5 per cent of ethanol, 1 per cent of acetic acid, and 7 lb. of a special medium for acetic acid bacteria, known as "aceto-pep" (devised by Frings).

OPERATION OF THE GENERATOR.—The mix is permitted to circulate repeatedly through the beechwood shavings until vinegar of the desired strength has been obtained. During the process heat is generated, and large quantities of oxygen are consumed. Since the tank is airtight, except for the air inlets and vent, the continued evolution of heat would produce a temperature sufficiently high to inhibit the action of the acetic acid bacteria. By passing the nutrient acid-alcohol mix through the cooling coils, the temperature of the mix is carefully controlled. The cooled mix is returned to the sparger and sprayed over the surface of the shavings. It trickles down through the shavings, acetic acid being formed from the ethanol. It is again cooled and recirculated. This cycle is repeated until the acetic acid content of the mix has become 105 grains. It may require 8 to 10 days for the conversion of the ethanol in a 2,500-gal. batch to acetic acid of this concentration. The vinegar is then withdrawn, except for about 200 gal., which is left as food for the bacteria and as a primer for the pump. A new batch of mix is promptly run into the generator.

ADVANTAGES OF THE PROCESS.—This process has several advantages. It may be operated at a low cost and it is easily controlled and relatively simple. Vinegars of higher acetic acid concentrations than those produced by other types of generators may be produced. The tank utilizes less space than that required by other types of generators for the production of the same quantity of vinegar in the same time. Losses due to evaporation and to the presence of fumes in the room are avoided, for the generator is airtight. The temperature of the room does not affect the temperature of the interior of the generator adversely. Owing to continuous operation, there is little tendency for slime to form in the generator.

Vinegar Generator with Automatic Control.—A vinegar generator system has been designed by Elmer[2] in which automatic control is exercised and in which the efficiency of yield is high. The vinegar mix flows by gravity from an elevated reservoir through a fluid course to the

[1] HANSEN, *loc. cit.*

[2] ELMER, L. S., Vinegar Generator System, U.S. Patent 2,156,428, 1939.

generator and thence to a storage reservoir. The fluid course is provided with a vessel for measuring the mix. A vertically adjustable float in the measuring vessel actuates a cock to cause the mix to flow in from the elevated reservoir. The mix is automatically discharged from the measuring vessel to the generator through the agency of a valve electrically actuated by an electric circuit regulated by a suitable clock. The temperature and rate of flow of air through the generator are regulated. For further details, consult the patent.

Revolving Generators.—Some vinegar is made by revolving generators.[1] These generators are essentially rotating drums or cylinders filled with shavings. The acidified nutrient alcoholic substrate is added in such quantity that the generator is approximately half filled (some drums hold 500 gal.). The cylinder is then caused to rotate slowly, possibly at 1.5 r.p.h., until vinegar of the desired acidity has been obtained, a process that may require about 3 weeks in some instances. Air, admitted through inlets, supplies oxygen to the upper portion of the drum. The slow rate of rotation causes the mix to become oxygenated. This method, according to Cruess, is not too popular, probably on account of the expense involved in constructing the drums and in operating them, also owing to their complexity.[1]

Acetic Acid.—In one commercial method for producing acetic acid, ethyl alcohol is converted in large wooden tanks, or generators, to a fairly pure dilute solution of acetic acid. Dilute alcohol is permitted to flow from a small wooden tub situated on top of the tank to a revolving arm, located just under the cover of the tank, which distributes it over beechwood shavings that have been impregnated with acetic acid bacteria. The solution trickles slowly down through the generator, through the bottom of which air enters. The acid is subsequently used in the production of acetate esters.

Crude acetic acid, or "pyroligneous acid," which is the principal source of commercial acid and acetates, is produced by dry distillation of certain kinds of hardwoods. Since it has no microbiological implications it need not be considered here.

Causes of Spoilage in the Vinegar Factory. *Vinegar Eels.*—Vinegar eels, *i.e.*, nematode worms (*Anguillula aceti*), may be a source of considerable trouble in vinegar factories, especially when the fruit from which the cider or wine is made has not been carefully controlled. They also gain access from dirt brought into the plant, and from insects. They may attack the bacterial film and cause it to sink and in some instances cause deterioration of the vinegar. They are harmless to human beings but from an aesthetic or quality standpoint are very objectionable in a

[1] Cruess, W. V., "Commercial Fruit and Vegetable Products," 2d ed., McGraw-Hill Book Company, Inc., New York, 1938.

product. Although quite small, about ⅛ in. long, they can readily be seen in a glass container by holding it before a strong source of light. In the factory they may be found around the edges of the surfaces of the liquid in barrels and in the generators. Their entrance can usually be prevented by keeping the plant in a high degree of cleanliness. Empty casks may be sulphured lightly to prevent their access. Once in the vinegar they can be destroyed by heating the vinegar to a temperature of about 130°F. (54°C.) or by pasteurization, and they can be eliminated by filtration, using Filter-Cel, or by fining. Infected barrels, tanks or generators may be treated with live steam.

Mites.—Mites breed rapidly in the presence of warmth and moisture. Cleanliness of a high order may be necessary to prevent mites from appearing in an establishment. By placing a ring of turpentine or some other viscid or repellent substance around each air hole in a cask or barrel, their access may be prevented. In order to eliminate mites the methods used against eels may be employed. The room in which they are found must be thoroughly cleaned and may be washed with an emulsion of kerosene and water. Fumigating the room with sulphur helps sometimes. Steam and hot water will destroy the mites.

Vinegar Flies.—Vinegar flies (species of *Drosophila*) breed in decayed fruit, fruit juices, and vinegar. By preventing these substances from being spilled about and by keeping the factory scrupulously clean their presence can usually be avoided. The placing of screens over the windows and doors of the establishment and the use of fine screens over the holes in barrels in which the fermentation is taking place are very helpful in keeping out these and other flies.

Wine Flowers.—"Wine flowers" is the term used by wine manufacturers to denote the whitish film, often much plicated, composed of yeast-like cells, which grows on the surface of wines or nutrient alcoholic solutions. This film is sometimes called *Mycoderma vini*. The organisms making up the film are strongly aerobic, grow very rapidly, and in the course of time will oxidize many of the carbon-containing constituents to carbon dioxide and water. Flavor and alcohol are thus destroyed, while the solution becomes cloudy. Wine flowers can be prevented by storing the alcoholic solution in completely filled and closed containers or tanks, or by adding 1 part of vinegar to 3 parts of the alcoholic solution.

Darkening of Vinegar.—Darkening of vinegar may be caused by iron and tannin or by an oxidase. As little as 1 part of iron in 10,000 parts of vinegar may cause a darkening of the product owing to the formation of iron tannates, if tannin is present in sufficient quantity. Iron is usually dissolved by the vinegar by contact with some iron source, while tannin may be extracted from new casks, especially oak, and also occurs in small quantity in the fruit juices. Aeration followed by fining satisfactorily

removes the darkening caused by iron and tannin. The special type of oxidase, an oxidizing enzyme which produces darkening in fruit juices, may be destroyed by pasteurizing the vinegar.

Yields.—Under favorable conditions 50 to 55 parts of acetic acid may be obtained from 100 parts of sugar, or approximately 1.26 g. of acetic acid from 1 g. of ethanol, according to Cruess. A portion of the sugar is consumed in the production of substances other than ethanol and as food by the yeast. There is also a loss usually of some alcohol and acetic acid by evaporation during the two fermentations.

Problem.—What yield of acetic acid can be obtained from 1 kg. of glucose, assuming 90 per cent efficiency in each of the conversion processes? How many grams and what volume (in liters) of air are theoretically required to convert the ethanol to acetic acid?

Solution:

$$1.\quad \underset{\text{Glucose}}{\overset{(180)}{C_6H_{12}O_6}} = \underset{\text{Ethanol}}{\overset{(2 \times 46)}{2C_2H_5OH}} + \underset{\substack{\text{Carbon}\\\text{dioxide}}}{\overset{(2 \times 44)}{2CO_2}} \quad \text{(Gay-Lussac equation)}$$

$$2.\quad \underset{\text{Ethanol}}{\overset{(2 \times 46)}{2C_2H_5OH}} + \underset{\text{Oxygen}}{\overset{(2 \times 32)}{2O_2}} = \underset{\text{Acetic acid}}{\overset{(2 \times 60)}{2CH_3COOH}} + 2H_2O$$

Theoretically, 1 mol of glucose (180 g.) will yield 2 mols, or 120 g., of acetic acid, which represents 2 parts of acetic acid from 3 parts of glucose. One kilogram of glucose would yield $(1{,}000/180) \times 120$ g. $= 667$ g. of acetic acid.

Assuming 90 per cent efficiency in both reactions (1) and (2), the yield of acetic acid would be $667 \times 0.9 \times 0.9 = 540$ g. This represents a yield of 54 parts of acetic acid from 100 parts of glucose.

From Eq. (2), 92 g. of ethanol would require 64 g. of oxygen for conversion to acetic acid. Assuming 90 per cent efficiency in Eq. (1), 460 g. of ethanol $[1000 \times (92/180) \times 0.9]$, would theoretically require $(460/92) \times 64 = 320$ g. of oxygen $= 224$ liters. Since air is approximately one-fifth oxygen by volume, the quantity of air required to convert 1 kg. of glucose to acetic acid would be 1,600 g. or 1,120 liters. As a matter of fact not all the oxygen in the air becomes fixed by the bacteria, and several times this volume should be available.

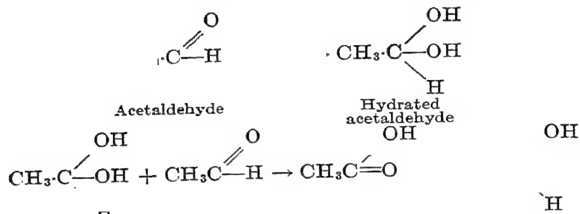
Grains Strength.—The term "grains strength" is commonly used to express the concentration of the acetic acid in a vinegar. One-grain vinegar contains 0.1 g. of acetic acid in 100 cc. at 20°C. (1 mg. per cc.). Vinegar containing 4 g. of acetic acid per 100 cc. at 20°C., approximately 4 per cent, is of 40 grains strength. In other words, the grain strength is ten times the acetic acid content in percentage.

Uses of Vinegar.—Vinegar is used for several purposes in connection with food: as a condiment for direct use on the table or on a commercial scale in the manufacture of mayonnaise, French dressing, pickles, relishes,

catsup, prepared mustard, and horse radish; for preventing mold growth in bread; and for other purposes. It may be used as an antiseptic.[1]

Mechanism of the Acetic Acid Fermentation.—In the acetic acid fermentation, acetaldehyde is an established intermediate product, having been first shown to be such by Hoyer (1899). Its fixation can be demonstrated with neutral calcium sulphite.

Under anaerobic conditions 1 molecule of acetaldehyde may act as the hydrogen acceptor for a second hydrated molecule of the same substance. The result is a Cannizzaro reaction in which 1 molecule of ethanol and 1 molecule of acetic acid are produced from 2 molecules of acetaldehyde, the ethanol being formed by hydrogenation of acetaldehyde and the acetic acid by dehydrogenation of the hydrated acetaldehyde:



Acetaldehyde Hydrated acetaldehyde

Hydrated acetaldehyde Acetaldehyde Acetic acid Ethanol

Neuberg and Windisch are of the opinion that ethanol is aerobically transformed to acetaldehyde, which in turn is dismutated, in the manner illustrated above, to equimolar quantities of ethanol and acetic acid. Alternate oxidation and dismutation follow until all the ethanol is converted to acetic acid.

Neuberg and Windisch[2] showed that *Acetobacter ascendens*, *A. pasteurianum* and *A. xylinum* were able to dismutate acetaldehyde to equimolar quantities of acetic acid and ethanol anaerobically. They likewise demonstrated that other aldehydes could be converted in a similar manner to their corresponding alcohols and acids.

Other workers[3] have shown that similar reactions may take place under anaerobic conditions.

It seems most likely[4] that in the normal acetic acid fermentation acetaldehyde is dehydrogenated to acetic acid. Oxygen acts as the

[1] McCulloch, E. C., "Disinfection and Sterilization," Lea & Febiger, Philadelphia, 1936.

[2] Neuberg, C., und F. Windisch, *Biochem. Zeit.*, **166**: 454 (1925).

[3] Wieland, H., und A. Bertho, *Ann.*, **467**: 98 (1928); Molinari, E., *Biochem. Zeit.*, **216**: 187 (1929).

[4] Butlin, K. R., "The Biochemical Activities of the Acetic Acid Bacteria," Chemistry Research, Special Report 2, H. M. Stationery Office, London, 1936.

hydrogen acceptor in the conversion of alcohol to acetaldehyde (a catalytic dehydrogenation):

$$\underset{\text{Ethanol}}{CH_3{\cdot}C\overset{\diagup OH}{\underset{\diagdown H}{—}}H} + O \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}C\overset{\diagup\!\!\diagup O}{—}H} + H_2O$$

Acetaldehyde is hydrated:

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}C\overset{\diagup\!\!\diagup O}{—}H} + H_2O \rightarrow \underset{\text{Hydrated acetaldehyde}}{CH_3{\cdot}C\overset{\diagup OH}{\underset{\diagdown H}{—}}OH}$$

Two of the hydrogen atoms of the hydrated acetaldehyde are activated and donated to oxygen, the hydrogen acceptor:

$$\underset{\text{Hydrated acetaldehyde}}{CH_3{\cdot}C\overset{\diagup OH}{\underset{\diagdown H}{—}}OH} + O \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}C\overset{\diagup OH}{=}O} + H_2O$$

When conditions become favorable some acetaldehyde may be converted to acetic acid by dismutation. In this case acetaldehyde becomes the hydrogen acceptor as well as the donator.

Substances other than oxygen and acetaldehyde may act as hydrogen acceptors, according to Wieland and Bertho.[1]

Methylene blue and benzoquinone are two such substances.

SOME OTHER FERMENTATIONS

The oxidations brought about by various species of the genus *Acetobacter* are of particular significance, since some of the compounds, such as ketoses and keto acids, formed from suitable substrates by these bacteria are prepared with considerable difficulty by purely chemical methods.

Species of the genus *Acetobacter* vary in their ability to oxidize or dehydrogenate various substances. Some species, for example *A. rancens*, oxidize a substrate to a high degree, sometimes forming carbon dioxide and water as the principal end products. Such bacteria, obviously, have no industrial value. Other species of the genus *Acetobacter* bring about the incomplete oxidation of a substrate and, accordingly, may be of much importance.

A. suboxydans is a species well-adapted for industrial use for it generally brings about the incomplete oxidation of sugars, alcohols, and acids even when a liberal supply of oxygen is available, as is essential for

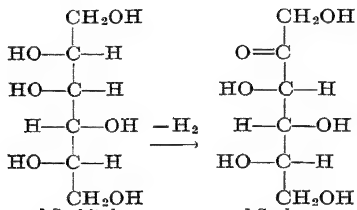
[1] *Loc. cit.*

a rapid dehydrogenation of the substrate. Butlin[1] and Kluyver and Boezaardt[2] have demonstrated, however, that the cells present in young cultures of *A. suboxydans* may produce some carbon dioxide from glucose.

A. xylinum, the sorbose bacterium, produces incomplete oxidation of substrates also, but in the presence of large quantities of oxygen may oxidize the substrates completely, forming carbon dioxide and water.[2]

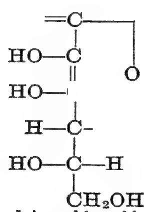
Some Products Formed by A. suboxydans.—In Table 53 are shown some of the products formed by *A. suboxydans* as the result of the oxidation of suitable substrates. These products are in most instances also produced by other species of the genus *Acetobacter.*

The Sorbose Fermentation.—*l*-Sorbose is a compound of especial interest on account of its use in the synthesis of vitamin C. By chemical means, *d*-sorbitol is produced from dextrose; by biological dehydrogenation, *d*-sorbitol is converted to *l*-sorbose:



d-Sorbitol *l*-Sorbose

The relation of *l*-sorbose to vitamin C (*l*-ascorbic acid) can be illustrated by showing the structural formula of this vitamin as assigned by Haworth and others:



l-Ascorbic acid
(Vitamin C)

It has been known since the latter part of the nineteenth century that certain oxidative bacteria have the ability to produce *l*-sorbose from *d*-sorbitol. Bertrand, in 1896, published the results of some of his researches with the sorbose bacterium, which was discovered by him, now known as *A. xylinum.* The organism with which he worked was called

[1] Butlin, K. R., *Biochem. Jour.,* **30**: 1870 (1936); **32**: 508 (1938).

[2] Kluyver, A. J., et A. G. J. Boezaardt, *Rec. trav. chim.,* **57**: 609 (1938).

the "sorbose bacterium" because it produced sorbose from the sorbitol present in the berries of the mountain ash (*Sorbus aucuparia*).

53.—SOME SUBSTRATES OXIDIZED BY *A. suboxydans* AND THE PRODUCTS FORMED

Substrate	Product formed	Substrate	Product formed
CH_2OH HOCH HOCH HCOH HCOH HOCH CH_2OH Perseitol	CH_2OH O═C HOCH HCOH HCOH HOCH CH_2OH Perseulose	CH_2OH HOCH HOCH HCOH HCOH CH_2OH *d*-Mannitol	CH_2OH O═C HOCH HCOH HCOH CH_2OH *d*-Fructose
CHO HCOH HOCH HCOH HCOH CH_2OH *d*-Glucose	COOH HCOH HOCH HCOH HCOH CH_2OH *d*-Gluconic acid	CH_2OH HCOH HCOH HCOH CH_2OH *i*-Adonitol	CH_2OH HCOH HCOH C═O CH_2OH "Adoninulose"
COOH HCOH HOCH HCOH HCOH CH_2OH *d*-Gluconic acid	COOH HCOH HOCH HCOH C═O CH_2OH *d*-5-Ketogluconic acid	CH_2OH HOCH HOCH CH_2OH Erythritol	CH_2OH O═C HOCH CH_2OH *l*-Erythrulose
COOH HCOH HOCH HCOH HCOH CH_2OH *d*-Gluconic acid	COOH C═O HOCH HCOH HCOH CH_2OH *d*-2-Ketogluconic acid	CH_3 HCOH HCOH CH_3 2:3-Butylene glycol	CH_3 HCOH C═O CH_3 Acetylmethylcarbinol
		CH_2OH HCOH CH_3 *α*-Propylene glycol	CH_2OH C═O CH_3 Acetol
CH_2OH HCOH HOCH HCOH HCOH CH_2OH *d*-Sorbitol	CH_2OH HCOH HOCH HCOH C═O CH_2OH *l*-Sorbose	CH_2OH HCOH CH_2OH Glycerol	CH_2OH C═O CH_2OH Dihydroxyacetone
		CH_2OH CH_2OH Ethylene glycol	CH_2OH COOH Glycollic acid

In general, species of the genus *Acetobacter* may be used to produce sorbose from sorbitol by oxidation. The most important of these are

A. suboxydans, *A. xylinum*, *A. rancens*, *A. aceti*, *A. melanogenum*, *A. gluconicum*, and *Bact. xylinoides*.

Sorbose may be prepared from sorbitol by two different methods of fermentation: by surface growth and by submerged growths. In this respect and in other ways this fermentation is similar to the gluconic acid fermentation.

Surface Growth.—In some of the earlier researches, sorbitol in concentrations of 2 to 6 per cent was used, the fermentations requiring 3 to 40 days for completion. Fulmer and his associates[1] devised a method of fermentation in which sorbitol in concentrations up to and including 35 per cent was readily fermented by *A. suboxydans* with high final yields. Yeast extract was used in the fermentation mashes as a nutrient in a concentration of 0.5 g. per 100 cc. The rate of sorbose formation decreased with an increasing concentration of sorbitol, but the percentage of final yield, based on the sorbitol originally present, varied but little over the range of concentrations from 5 to 35 per cent. A 40 per cent concentration of sorbitol markedly reduced the activity of *A. suboxydans*. The temperature range of 25 to 30°C. was optimum for the fermentation.

The ratio of surface to volume (square centimeters of area per 1 cc. volume) was important in this fermentation. The final yield, likewise the rate of sorbose formation, increased (within limits) with an increasing surface-volume ratio.

Yields of over 80 per cent of sorbose were obtained from sorbitol by the foregoing procedure. If the bacterial films or mats were disturbed in the flasks, the yields of sorbose were reduced.

Submerged Growth.—Production of sorbose from sorbitol by submerged growths in rotary drum fermenters has been successfully carried

TABLE 54.—THE EFFECT OF CONCENTRATION OF *d*-SORBITOL ON PRODUCTION OF *l*-SORBOSE BY SUBMERGED GROWTHS OF *Acetobacter suboxydans*[1]

[Air flow, 1,800 cc. per min.; r.p.m., 13; gauge pressure, 30 lb. per sq. in. (2.11 kg. per sq. cm.)]

Sorbitol concentration, per cent	Fermentation period, hours	Total sorbitol available, grams	Sorbose produced	
			Grams	Per cent by weight
10.1	16.5	332	307	92.5
14.5	24	453	423	93.4
19.8	45*	632	617	97.6
30.3	69	975	194	19.9

[1] WELLS, P. A., J. J. STUBBS, L. B. LOCKWOOD, and E. T. ROE, *Ind. Eng. Chem.*, **29**: 1385 (1937).
* Fermentation practically completed in 33 hr.

[1] FULMER, E. I., J. W. DUNNING, J. F. GUYMON, and L. A. UNDERKOFLER, *Jour. Am. Chem. Soc.*, **58**: 1012 (1936).

out by Wells, Stubbs, Lockwood, and Roe[1] of the Industrial Farm Products Research Division, Bureau of Chemistry and Soils, U.S. Department of Agriculture. The procedure used by these men was similar to that employed in the drum method for gluconic acid production (refer to Chap. XXVI). Good results were obtained when the concentration of sorbitol was 20 per cent, the air flow 1800 cc. per min., the gauge pressure 30 lb. per sq. in., and the drum rotation 13 r.p.m. Fermentations were carried out at 30°C., using yeast extract in the proportion of 5 g. per liter as the only added nutrient material.

An examination of the preceding table shows that a 97.6 per cent yield of sorbose was obtained from a 19.8 per cent concentration of sorbitol in practically 33 hr. under the conditions of the fermentation. The advantages of this type of fermentation in comparison with the surface-growth type are apparent.

An active inoculum was prepared by growing *A. suboxydans* in a solution containing 100 g. of sorbitol, 5 g. of Difco yeast extract, 10 g. of glucose, and 3.1 g. of calcium carbonate per liter of solution. Calcium carbonate is a necessary component of this solution, for the gluconic acid formed from glucose, unless neutralized, will prevent sorbose from being produced. More uniform results were obtained from the inoculum when using conditions of air flow, pressure, and agitation that did not favor a quick oxidation of sorbitol.

2 TUBE CULTURES OF ORGANISM

TO INOCULATE ↓

800 CC. 10% SORBITOL, 0.5% DIFCO YEAST EXTRACT SOLUTION AERATION 48 HOURS IN BOTTLE

800 CC. TO INOCULATE

2400 CC. NUTRIENT SOLUTION CONTAINING SORBITOL, YEAST EXTRACT, GLUCOSE, AND CALCIUM CARBONATE IN DRUM FERMENTER LIMITED AERATION AND AGITATION AT ATMOSPHERIC PRESSURE FOR 24 TO 48 HOURS

100 CC. TO INOCULATE

3200 CC. SORBITOL SOLUTION TO BE FERMENTED IN DRUM FERMENTER

OXIDATION ↓

SORBOSE-YIELDS IN EXCESS OF 90% IN 16 TO 32 HOURS

Fig. 33.—Diagram of large-scale inoculum preparation method for producing sorbose from sorbitol. [*Courtesy P. A. Wells, J. J. Stubbs, L. B. Lockwood, and E. T. Roe, Ind. Eng. Chem.*, **29**: 1385 (1937).]

Semiplant-scale Production.—The production of *l*-sorbose from *d*-sorbitol by *A. suboxydans* has been carried out successfully on a semiplant scale substituting corn steep liquor (3 g. per liter) for dried yeast extract as the only source of nutrient material.[2] It was necessary, however, to use octadecyl alcohol, an antifoam substance, to prevent excessive frothing of the medium.

Control of the pH was accomplished by adding to the nutrient medium an amount of calcium carbonate slightly in excess of that required to

[1] Wells, P. A., J. J. Stubbs, L. B. Lockwood, and E. T. Roe, *Ind. Eng. Chem.*, **29**: 1385 (1937).

[2] Wells, P. A., L. B. Lockwood, J. J. Stubbs, E. T. Roe, N. Porges, and E. A. Gastrock, *Ind. Eng. Chem.*, **31**: 1518–1521 (1939).

neutralize the corn steep liquor and the gluconic acid produced by fermentation from the small quantity of glucose present in the sorbitol sirup used.

Sorbitol, in concentrations as high as 30 per cent, was oxidized to *l*-sorbose rapidly and efficiently. It required a longer period of time to oxidize the higher concentrations of *d*-sorbitol; for example, in one case a 10 per cent sorbitol solution required only 13.5 hr. while a 30 per cent solution required 45 hr. for the conversion of most of the sorbitol to sorbose.

Results of one large-scale experiment are given in Table 55.

TABLE 55.—TYPICAL PILOT-PLANT-SCALE RESULTS ON THE PRODUCTION OF *l*-SORBOSE FROM *d*-SORBITOL BY *A. suboxydans*[1]

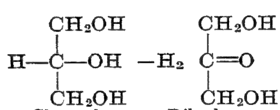
Total sorbitol available (dry basis), kg	163.4
Total sorbose produced, kg	151.0
Total sorbose recovered (3 crops), kg	114.3
Weight yield based on sorbose produced, per cent	92.4
Weight yield based on sorbose recovered, per cent	69.8

[1] WELLS, P. A., L. B. LOCKWOOD, J. J. STUBBS, E. T. ROE, N. PORGES, and E. A. GASTROCK, *Ind. Eng. Chem.*, **31**: 1518–1521 (1939).

Use of Vats.—Vats are used in the commercial production of sorbose.

Maintenance of Stock Cultures.—Stock cultures of *A. suboxydans* be carried on agar slants containing 5 per cent sorbitol or on malt-extract agar slants.

The Production of Dihydroxyacetone from Glycerol.—Dihydroxyacetone is an industrially important chemical compound formed biologically through dehydrogenation, or oxidation, of glycerol by certain acetic acid bacteria:



Glycerol Dihydroxyacetone

In the preceding equation, oxygen is the hydrogen acceptor. Provided that they are sufficiently active, other hydrogen acceptors may be substituted for oxygen.

The formation of dihydroxyacetone by microorganisms was first observed by Bertrand. His sorbose bacterium (*A. xylinum*) oxidized glycerol to dihydroxyacetone. Since the original work by Bertrand, considerable research has been undertaken and several patents have been issued concerning the fermentation.

Organisms.—Various species of the genus *Acetobacter* may be used in the production of dihydroxyacetone from glycerol, but *A. suboxydans* and *A. xylinum* are most frequently employed. *Bacterium orleanense* and *A. aceti* have produced high yields of dihydroxyacetone. *Bact. xylinoides*, *A. gluconicum*, *A. dihydroxyacetonicum*, *A. melanogenum*, and *A. rancens* likewise form dihydroxyacetone from glycerol.

Optimum Conditions for Fermentation.—A suitable supply of nutrient material is necessary for a successful yield of dihydroxyacetone. Five per cent yeast water was used by Bertrand and some later investigators, but it has been replaced by the use of 0.5 per cent concentrations of yeast extract. Underkofler and Fulmer[1] obtained optimum yields of dihydroxyacetone when using 0.10 to 0.30 per cent of potassium dihydrogen phosphate in addition to 0.5 per cent yeast extract. Malt extract and other products have proved satisfactory as substitutes for yeast extract.

Aeration, temperature, pH, and glycerol concentration are important factors. Visser't Hooft[2] investigated the effect of aeration on glycerol transformation and obtained the following data in connection with his study involving *A. suboxydans*.

TABLE 56.—EFFECT OF AERATION ON THE OXIDATION OF GLYCEROL[1]

Time	Glycerol transformed to dihydroxyacetone, per cent	
	With aeration	Without aeration
After 2 days	35	11
After 4 days	84	30
After 6 days	90	57

BUTLIN, K. R., "The Biochemical Activities of the Acetic Acid Bacteria," Chemistry Research, Special Report 2, H. M. Stationery Office, London, 1936.

Bernhauer and Schön found a medium 1 to 2 cm. deep to be optimum from the viewpoint of aeration.

In Table 57 are summarized some data concerning the optimum temperature, pH, and glycerol concentration for the conversion of glycerol to dihydroxyacetone by certain specified acetic acid bacteria.

Underkofler and Fulmer found that glycerol concentrations greater than 6 per cent produced lower yields of dihydroxyacetone.

Yields.—Yields of 85 to 95 per cent of dihydroxyacetone on the basis of the glycerol fermented are not uncommon.

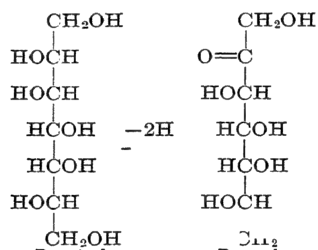
[1] UNDERKOFLER, L. A., and E. I. FULMER, *Jour. Am. Chem. Soc.*, **59**: 301 (1937).

[2] VISSER'T HOOFT, F., "Biochemische onderzoekingen over het geslacht *Acetobacter*," Thesis, Technische Hoogeschool, Delft, 1925.

TABLE 57.—SOME OPTIMUM CONDITIONS FOR THE OXIDATION OF GLYCEROL TO DIHYDROXYACETONE

Organism	Optimum temperature, °C.	Optimum	Optimum glycerol concentration, per cent	Investigators
Organism closely related to *A. xylinum*	27–28	4–4.8	6–8	Bernhauer und Schön (1928)
A. xylinum			2	Cozic (1933)
A. xylinum; A. dihydroxyacetonicum				Virtanen und Bärlund (1926)
A. suboxydans.	28–30	5.5–7.0		Underkofler and Fulmer (1937)

The Perseulose Fermentation.—Perseulose is a rare ketoheptose. It may be prepared by the oxidation or dehydrogenation of perseitol, an alcohol that occurs as a constituent of the avocado (*Persea gratissima*):



Perseitol Perseulose

Bertrand[1] discovered perseulose in 1908, as a product of the oxidation of perseitol by the sorbose bacterium, *A. xylinum.*

Tilden[2] has carried out research, in cooperation with Wells, Stubbs, Lockwood, and Roe, concerning the production of perseulose from perseitol, using *A. suboxydans.*

In general, best results were obtained by Tilden when the medium contained 0.3 per cent of neutralized corn steep liquor (yeast extract, in 0.5 per cent concentration, may be used in place of neutralized corn steep), 0.3 per cent KH_2PO_4, 0.05 per cent glucose (promoted complete oxidation of perseitol), and 3 to 4 per cent of perseitol.

D, G., *Compt. rend.*, **147**: 201 (1908).

[2] TILDEN, E. B., *Jour. Bact.*, **37**: 629–637 (1939).

Using rotary-drum fermenters, of the type described in Chap. XXVI, and conditions similar to those found most favorable for the production of sorbose from sorbitol (the use of 3,200 cc. of culture per drum, an air flow of 1,200 cc. per min., a pressure of 30 lb., and a rotation speed of 13 r.p.m.), 3 to 4 per cent concentrations of perseitol were oxidized quantitatively to perseulose in 36 to 48 hr. at a temperature of 30°C.

At a later time, a pH of 6.4 was found to be optimum for the fermentation, instead of the pH of 5.5, or less, which was used in the rotary-drum fermenters. Thus the time required for the conversion may have been reduced still further had the optimum pH been used.

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CHAPTER XVI

THE PRODUCTION OF LACTIC ACID BY FERMENTATION

Lactic acid, or alpha-hydroxypropionic acid ($CH_3 \cdot CHOH \cdot COOH$) as an unnamed component of soured milk must have been known in human experience since the days when man first had his flocks and herds. Its true nature was discovered by Scheele, who isolated and identified it as the principal acid in sour milk in 1780.

Lactic acid was first recognized as a fermentation product by Blondeau in 1847. It was investigated by Pasteur as one of his first microbiological problems. Schultze (1868) demonstrated the presence of lactic acid bacteria in yeast cultures of distilleries. But it was not until the year 1877 that lactic acid bacteria were isolated in pure cultures, Dr. Lister having isolated *Streptococcus lactis*. During this same period Delbruck was endeavoring to determine the most favorable temperature for lactic acid fermentation in distilleries. He concluded that relatively high temperatures favored high yields of lactic acid.

Avery, of Littleton, Mass., was the first person (1881) to produce lactic acid successfully on a commercial basis. At that time an effort was being made to substitute calcium lactate for the tartrates then being used in baking powders. The attempt at replacement was unsuccessful but many new uses for the acid were found. Since 1881 the production of lactic acid by fermentation has become a very important industry. Lactic acid is now produced commercially from corn sugar, molasses, and whey.

Forms of Lactic Acid.—Lactic acid occurs in three forms: levorotatory lactic acid, dextrorotatory lactic acid (known also as "sarcolactic acid"), both of which are optically active acids, and *i*-lactic acid, an optically inactive acid.

Lactic acid of various forms is produced by the lactic acid bacteria. *Lactobacillus delbrueckii* and *S. lactis* usually produce *d*-lactic acid, while *L. leichmannii* and *Leuconostoc mesenteroides* var. Sake commonly produce *l*-lactic acid. A few bacteria produce *i*-lactic acid, for example, *Lactobacillus pentoaceticus* (in "Bergey's Manual of Determinative Bacteriology"[1] this organism is listed as a probable synonym of *L. brevis*).

The lactic acid produced during fermentation is frequently inactive. Pederson[2] and others and later Tatum[3] and his associates showed that

[1] 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

[2] Pederson, C. S., W. H. Peterson, and E. B. Fred, *Jour. Biol. Chem.*, **68**: 151 (1926).

[3] Tatum, E. L., W. H. Peterson, and E. B. Fred, *Biochem. Jour.*, **26**: 846 (1932).

Clostridium acetobutylicum caused lactic acid bacteria, which usually formed active lactic acid, to produce inactive lactic acid. It was suggested by Tatum and his associates[1] that racemization might be brought about by an enzyme system elaborated by *Cl. acetobutylicum* and *Cl. butylicum.* It has been shown by Katagiri and Kitahara[2] that the enzyme racemiase is responsible for the conversion of optically active lactic acids to inactive lactic acids. Thus the contaminants found in a fermentation medium may under certain circumstances be responsible for the racemization of active lactic acid.

Classification of Lactic Acid Bacteria.—A large number of bacteria produce lactic acid from carbohydrate materials. Many have no industrial significance, however.

In one method of classification the true lactic acid bacteria may be divided into two groups. One group is made up of those bacteria which convert carbohydrate materials to lactic acid as the principal end product. *L. delbrueckii* is an example of this group. Kluyver and Donker have suggested that this group be called the "homofermentative" lactic acid bacteria in contrast to a second group of lactic acid bacteria which produces, in addition to lactic acid, volatile acids and carbon dioxide in quantity, and for which they propose the name "heterofermentative." The latter group includes such bacteria as *L. lycopersici*, *L. mannitopoeus* and *L. acidophil-aerogenes*. Lactic and acetic acids, ethanol, glycerol, and carbon dioxide are the main end products formed by these bacteria.

In a second method of classification, the lactic-acid-forming bacteria may be grouped, according to their significance to man, into organisms of industrial importance, of which *L. delbrueckii*, *L. bulgaricus* and *Strept. lactis* are examples; organisms of possible therapeutic significance, such as *L. acidophilus;* and organisms of sanitary significance, such as *E. coli.*

THE COMMERCIAL PRODUCTION OF LACTIC ACID

Some General Considerations. *Organisms Used.*—The organisms that may be used for the production of lactic acid by fermentation are *L. delbrueckii*, *L. casei*, *L. leichmannii*, *L. bulgaricus* and *Strept. lactis.* All these organisms are homofermentative.

The type of organism selected for a fermentation depends primarily upon the carbohydrate being fermented and the temperature to be used. *L. bulgaricus*, *L. casei*, or *Strept. lactis*, may be used to ferment milk or whey, *L. bulgaricus* being favored. In the fermentation of dextrose or maltose *L. delbrueckii*, *L. leichmannii*, or *L. bulgaricus* may be used. Frequently *L. delbrueckii* is used with another lactic acid producer, such as *L. bulgaricus* or *Strept. lactis*, to ferment hydrolyzed starches.

[1] *Ibid.*, **30**: 1892 (1936).

[2] KATAGIRI, H., and K. KITAHARA. Refer to the papers cited at the end of the chapter.

Carbohydrates Suitable for Utilization.—A large number of carbohydrates may be utilized for lactic acid production. The acid is generally produced from glucose, sucrose, or lactose. Starches, corn and potato especially, may be hydrolyzed by enzymes, or by acids (preferably sulphuric acid) to maltose and glucose. Xylose is fermented by *L. pentoaceticus* to yield lactic acid and acetic acid, chiefly. Molasses and whey are generally low-priced sources of sugars for the fermentation. Smith and Claborn[1] have estimated that 2,700,000,000 lb. of lactose are obtained from skim milk, buttermilk, and whey annually. Of this quantity, a large percentage could be used in lactic acid production. The choice of carbohydrate used will depend upon its availability; fermentability, with or without preliminary treatment; and its cost. In this country, corn sugar, molasses, and whey are much used; in Germany, potato starch.

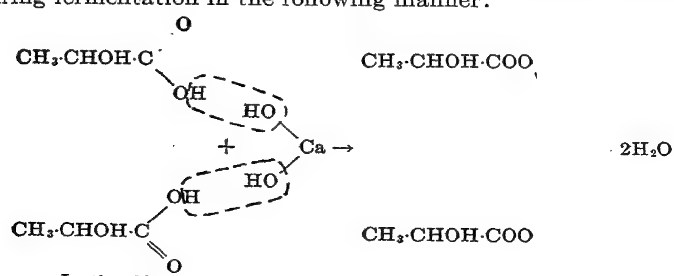
Temperature of the Fermentation.—The lactic acid fermentation is carried out at comparatively high temperatures. In fermentations using *L. delbrueckii* a temperature of 45°C., or higher, may be maintained. *L. bulgaricus* may be incubated at 45 to 50°C.; *L. casei*, or *Strept. lactis*, at about 30°C. The optimum temperature should be determined experimentally for each type of fermentation.

Concentration of Sugar.—The sugar in mashes is normally adjusted to a concentration of 5 to 20 per cent, depending on the nature of the raw material and the conditions of the process.

Oxygen Relationship.—The bacteria used to produce lactic acid industrially are usually microaerophilic or anaerobic in nature. *Strept. lactis* is listed as a facultative aerobe.

pH.—The fermentation proceeds best when the pH is on the acid side of neutrality. Owing to the addition of calcium carbonate, calcium hydroxide, or some other neutralizing agent to the fermentation mash, the pH tends to approach neutrality. The pH may be maintained at a constant value by the use of ammonia as the neutralizing agent.

Neutralization of Acid.—Lactic acid is neutralized by calcium hydroxide during fermentation in the following manner:



Lactic acid Calcium hydroxide Calcium lactate

[1] Smith, L. T., and H. V. Claborn, *Ind. Eng. Chem.* (*News Ed.*), **17**: 370 (1939).

If the lactic acid were not neutralized, the lactic acid bacteria would not be able to tolerate the high acidity developed and the fermentation would not continue to completion.

Calcium (or zinc) hydroxide or carbonate may be added either at the beginning of the fermentation or intermittently as the fermentation progresses. Peterson, Fred, and Davenport suggested that the preliminary introduction of a neutralizing agent was as efficacious as intermittent introduction from the point of view of the speed and completeness of the conversion of xylose to lactic acid. The advantage of adding the carbonate intermittently lies in the fact that an acid reaction helps to prevent contaminants from gaining ascendency during the fermentation.

Growth Factors for Lactic Acid Bacteria.—Certain growth factors appear to be essential for certain lactic acid bacteria. Orla-Jensen[1] and his fellow workers reported that riboflavin and at least one other "activator" were required by certain lactic acid bacteria for normal development. Wood and his associates[2] confirmed their findings in respect to riboflavin.

Snell, Strong, and Peterson[3] described the preparation of an active concentrate from liver extract, which was essential for the normal growth of the species of lactic acid bacteria investigated by them. It has been shown that this active substance was pantothenic acid.[4,5]

Nicotinic acid stimulated growth and acid production by some lactic acid bacteria.[5]

For further information concerning this important subject, the reader is referred to the foregoing citations and others listed at the end of the chapter.

Accessory Nutrients in the Fermentation of Molasses.—Stiles and Pruess[6] have shown that good yields of lactic acid and a short fermentation period result from the addition of such accessory nutrients as malt sprouts, steep water, and thin grain residue to blackstrap molasses. The yield and fermentation time depend on the kind, quantity, and combination of the accessory nutrients.[6] It was suggested that the accessory nutrients supplied soluble organic nitrogen and stimulatory substances that were useful biologically.

[1] ORLA-JENSEN, S., N. C. OTTE, und A. SNOG-KJAER, *Centr. Bakt. Parasitenk.*, Abt. II, **94**: 434 (1936).

[2] WOOD, H. G., A. A. ANDERSEN, and C. H. WERKMAN, *Proc. Soc. Exptl. Biol. Med.*, **36**: 217–219 (1937).

[3] SNELL, E. E., F. M. STRONG, and W. H. PETERSON, *Biochem. Jour.*, **31**: 1789–1799 (1937).

[4] SNELL, E. E., F. M. STRONG, and W. H. PETERSON, *Jour. Am. Chem. Soc.*, **60**: 2825 (1938).

[5] SNELL, E. E., F. M. STRONG, and W. H. PETERSON, *Jour. Bact.*, **38**: 293–307 (1939).

[6] STILES, H. R., and L. M. PRUESS, *Jour. Bact.*, **36**: 149–153 (1938).

Duration of Fermentation.—A fermentation is usually complete in from 42 hr. to 6 days.

Yields.—Yields of 90 per cent on the basis of the sugar fermented are not uncommon in controlled processes. Higher yields have been obtained occasionally. Tatum and Peterson[1] have reported a yield of 100.5 g. of *d*-lactic acid from 100 g. of glucose.

Grades of Lactic Acid.—There are at least four principal grades of lactic acid. These include crude lactic acid of 22, 44, and 50 per cent strengths, edible lactic acid of 44 and 50 per cent strengths, plastic grade lactic acid of greater than 50 per cent strength, and U.S.P. grade lactic acid of 85 per cent strength.[2]

Standards may be set up by the consumers, the requirements depending on the use to be made of the lactic acid. Color, flavor, and odor are important, in addition to the ash content. For example, lactic acid used in the manufacture of transparent phenolic resins must be of high purity. The chloride, sulphate, and ash contents must be very low, especially the iron.

Lactic Acid Production by Continuous Fermentation.—A procedure for producing lactic acid from the lactose of sweet whey on a semiplant basis by continuous fermentation was worked out by Whittier and Rogers.[3] The main equipment used in this procedure included a storage tank for whey; a lime reservoir and feeding device; an insulated and covered fermentation tank with facilities for maintaining a constant temperature of 43° ± 0.1°C. and for agitating the medium at a slow rate; a storage tank to receive the overflow from the fermentation tank, which should have a capacity for 25 per cent of the latter tank; a coagulation tank, which should have 50 to 100 per cent of the operating capacity of the fermentation tank, and which is connected by way of a filter press or centrifuge to an evaporator.[4]

For operation, the fermentation tank is filled with sweet whey at a temperature of 43°C. The mash is inoculated with a culture of lactobacilli, for example, *L. bulgaricus* or *L. casei*, and sometimes with a yeast, which by reason of its associated growth accelerates the fermentation. The inoculated medium is agitated and maintained at 43°C. (as closely as possible) for the duration of the fermentation.

After the pH of the mash has dropped to 5.0, usually after approximately 12 hr., lime is fed into it to maintain its pH between 5.0 and 5.8, a range favorable to the lactic acid bacteria but inhibitory to the

[1] Tatum, E. L., and W. H. Peterson, *Ind. Eng. Chem.*, **27**: 1493 (1935).
[2] Smith, L. T., and H. V. Claborn, *Ind. Eng. Chem.* (*News Ed.*), **17**: 641 (1939).
[3] Whittier, E. O., and L. A. Rogers, *Ind. Eng. Chem.*, **23**: 532 (1931).
[4] *Ibid.*

development of contaminating organisms under the conditions of the fermentation.

At the end of 24 hr., and thereafter every 12 hr., the lactose content of the whey is ascertained. When the amount of lactose has become diminished to less than 1 per cent, usually 48 to 72 hr. after the mash has been inoculated, whey, which may be pretreated with lime to inhibit the development of bacteria, is introduced into the fermentation tank from the whey storage tank. The whey is introduced at such a rate that the volume fed during 24 hr. equals the volume of the fermentation tank.[1] The rate is then adjusted for the most efficient operation. In this connection, the lactose content of the overflowing whey should be determined.

The fermented whey is boiled until the proteins are entirely coagulated. If lactic acid is desired, rather than calcium lactate, sulphuric acid is added to precipitate the calcium and liberate free lactic acid. The coagulated protein and calcium sulphate are then separated by filtration from the lactic acid, which is concentrated in evaporators to the desired concentration.

Lactic Acid from Whey.—Lactic acid has been produced commercially from lactose since the year 1936 at Norwich, New York, in a plant operated by the Sheffield By-Products Company (a subsidiary of the Sheffield Farms, which in turn is a subsidiary of the National Dairy Products Corporation of New York). The plant has a capacity for 10,000 lb. of 22 per cent lactic acid per day. Technical and U.S.P. grades of lactic acid, calcium lactate, and sodium lactate are manufactured from whey.[2]

The utilization of casein whey has always been a problem. It may be dried and used as a feed for cattle and poultry, or the lactose and albumin may be recovered from it. Now, it may be used in the commercial production of lactic acid.

Surplus milk and skim milk from other sources may be used in the manufacture of lactic acid. The cream is removed from the milk and the casein precipitated by the use of lactic acid or hydrochloric acid, the former acid being preferred, for it may be recovered subsequently. The whey, known as casein whey, which is the product left after the separation of the cream and casein from the milk, contains lactalbumin (a protein), approximately 4.6 per cent lactose, vitamin G, mineral salts, and water. This medium serves as the nutrient substrate in the manufacture of lactic acid.

The commercial process for the production of lactic acid from the lactose of whey is based on the researches of Rogers and his associates of

[1] *Ibid.*

[2] Olive, T. R., *Chem. & Met. Eng.*, **43**: 480–483 (1936).

the Bureau of Animal Industry, U.S. Department of Agriculture. Chappell and his associates of the Sheffield By-Products Company and others

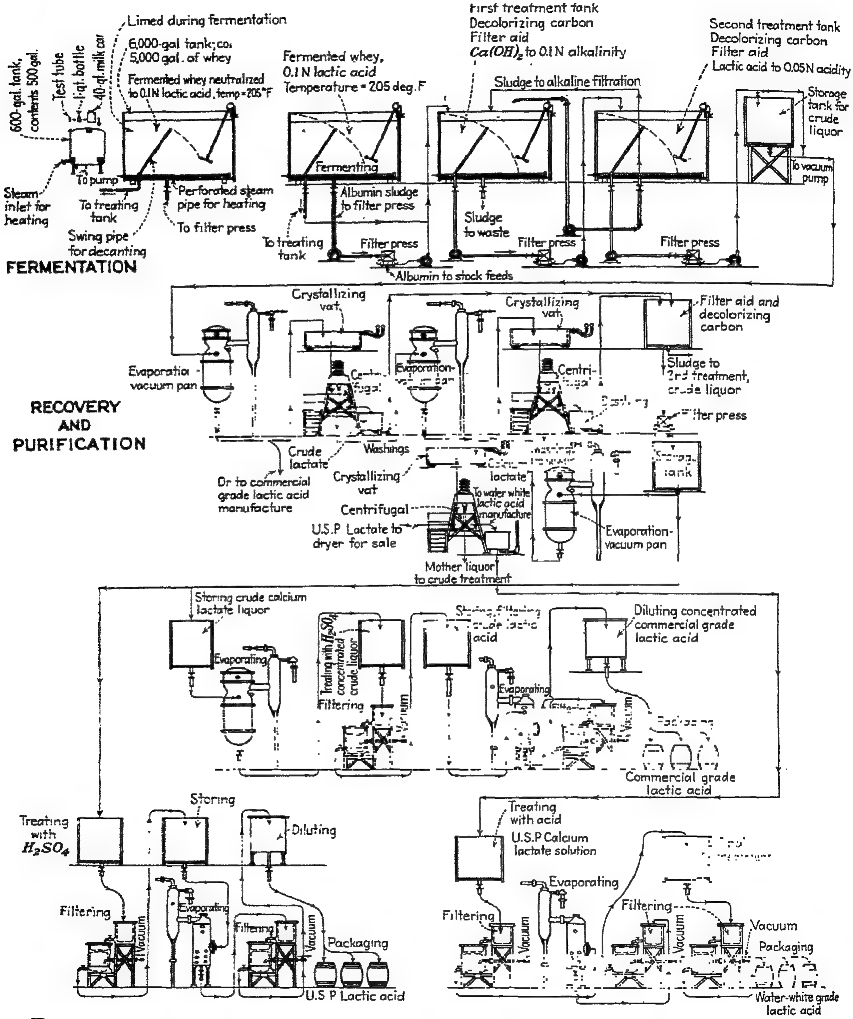


FIG. 34.—Lactic acid from casein whey. (*Courtesy L. V. Burton, Food Industries.*)

of the research laboratories of the National Dairy Products Corporation at Baltimore were largely responsible for making the process a commercial

one. This process has been described by Olive[1] and Burton.[2] The descriptions that follow are based on the articles by Dr. L. V. Burton, editor of *Food Industries*.

The Process in Brief.—Pasteurized whey is inoculated with a starter containing *L. bulgaricus*. During the fermentation the lactic acid produced from lactose is neutralized intermittently with lime. At the end of the fermentation, the lactalbumin is coagulated by heat. After permitting the coagulated lactalbumin to settle, the solution of calcium lactate is decanted off, filtered, treated with decolorizing carbon and filter aids, filtered, and evaporated. Crystallization follows. The crystals are washed, perhaps further purified, dried, and may be sold as calcium lactate or converted to lactic acid. Various procedures are followed in producing the different grades of lactates and lactic acid.

Preparation of the Starter.—The starter is prepared as follows. A quart of sterilized skim milk is inoculated with a culture of *L. bulgaricus*, containing also a yeast that causes the fermentation to become more vigorous. After incubation for 24 hr. at a temperature of 43°C. (110°F.), the contents of the bottle are placed in a 40-qt. jug containing pasteurized skim milk. The contents of the jug are dumped, after incubation at a temperature of 43°C. for 24 hr., into a glass-lined steel tank containing 500 gal. of pasteurized whey. Following incubation at 43°C. for 24 hr., this starter is added to the main fermentation tank (see Fig. 34).

The Fermentation Tank.—This tank is constructed of wood and has a capacity for 5,000 gal. of whey. In the bottom of the fermenter is a perforated brass pipe, through which steam may be introduced to warm the mash to the temperature desired for the fermentation or for the subsequent coagulation of lactalbumin. The tank is provided with a mechanical stirrer and a 4-in. brass dip pipe, which may be raised or lowered in order to decant the clear solution of calcium lactate, which separates after the coagulation of lactalbumin. Before use, the tank is cleaned, treated with a chemical agent to destroy microorganisms and subsequently rinsed with pure water. An outlet is located at the bottom of the tank for use in connection with its cleaning.

The Fermentation.—The inoculated whey is maintained at a temperature of 43°C. until the end of the fermentation, which is usually complete in about 42 hr. Lime, $Ca(OH)_2$, in the form of a slurry, is added to the fermenting mash every 6 hr. to keep the acidity of the mash below 0.6 per cent. By neutralizing the lactic acid with lime, the fermentation time is shorter and the yields are higher, for under these circumstances the bacteria are not inhibited by the acid that they produce. At the completion of fermentation, the mash is neutralized to 0.1 per cent lactic acid.

[2] Burton, L. V., *Food Industries*, **9**: 571, 634 (1937).

Filtration of the Fermented Mash.—The fermented medium, which is of a pale-green[1] color, is heated to 96°C. (205°F.) to coagulate the lactalbumin. The coagulated albumin is permitted to settle. Only a short time is necessary, however, for in about 10 min. the supernatant may be decanted.

The supernatant liquor is withdrawn through the decanting pipe and forced, by means of a centrifugal pump, through a plate-and-frame type of filter press to a storage tank. Here it is treated with lime to adjust the alkalinity to 0.1 per cent. A filter aid, such as diatomaceous earth, and a decolorizing carbon (Norit) are added. The mixture is thoroughly agitated and then the contents are permitted to stand. After approximately 15 min. the clear supernatant is decanted off and pumped through a filter press. The sludge is discarded. The clear solution, pumped to a clean wooden tank, is acidified to 0.05 per cent acid with lactic acid. Filter aid and decolorizing carbon are again added, settling is permitted, and the supernatant liquor is decanted and filtered. Sludge from this second treatment may be used to treat a fresh lot of calcium lactate solution.

Evaporation, Crystallization, and Washing.—The filtrate, or crude liquor, may be stored in a clean wooden tank (Fig. 34), or it may be concentrated in an evaporator, under a vacuum of 25 in., to a concentration of 15°Bé. and then pumped to crystallizing pans (equipped with casters to facilitate their being moved about) located on a gallery above the floor containing the evaporators. The crystallizing vats each have a capacity for 300 gal. Each is provided with a water jacket and the inner wall, which comes in contact with the calcium lactate, has a lining of stainless steel. Cooling water is passed through the jackets of the pans. Calcium lactate crystallizes out after 10 to 12 hr. at 10 to 15.6°C. (50 to 60°F.). The pans are pushed to chutes and the crystallized material shoveled down them to the baskets of Hepworth centrifugals. The baskets are spun. The mother liquor, which passes off, is reserved for further purification treatment. The crystals of calcium lactate that remain are washed with water, while the baskets are still spinning. The wash water thus obtained is evaporated to 13.5°Bé. and recrystallized, and the new crop of crystals is centrifuged and washed. The wash water is again evaporated, crystallization takes place, and the crystals are washed, the wash water being discarded this time.

From this series of treatments, three sets of crystals have resulted. These are combined, placed in a glass-lined tank, and dissolved in a small amount of water at 66°C. (150°F.). Norit and filter aid are mixed with the crude calcium lactate solution, the Norit for improving the color, and the filter aid for removing finely suspended particles during filtration. The supernatant is pumped to a filter press, while the sludge is returned

d., **9**: 571 (1937).

for the second treatment of the crude liquor. The thus refined liquor may be concentrated to 11.5°Bé. and then placed in vats for crystallization. Crystallization proceeds slowly, with the result that the crystals are purer. The crystals are washed, the washings being returned to the crude liquor, and then may be dried in a tunnel dryer. Such crystals constitute the U.S.P. grade of calcium lactate. The washed crystals, without drying, may be used in the manufacture of the best grades of lactic acid.

Grades of Lactic Acid.—Commercial, edible, and water-white grades of lactic acid are manufactured by the Sheffield By-Products Company.

LACTIC ACID OF COMMERCIAL GRADE.—This grade of lactic acid is produced from the crude calcium lactate liquor obtained after the first vacuum treatment (Fig. 34). This liquor, which has a concentration of 13.5°Bé., is pumped to a wooden tank. Decolorizing carbon and chemical agents for precipitating iron and heavy metals are added. The mix is made slightly acid with an electrolytic grade of sulphuric acid and pumped through a rubber connection to the upper tank of a vacuum filter made of stoneware. Calcium sulphate and other precipitated matter are removed. The precipitates are washed while on the filter. The filter cake is discarded, the filtrate and washings constituting a crude lactic acid solution of approximately 22 per cent strength.

The crude lactic acid may be stored in a wooden tank or further purified at once. The next step in the purification process is to concentrate the crude acid to 22°Bé. (50 to 60 per cent lactic acid) in a stainless-steel vacuum evaporator. The concentrated acid is conveyed to the upper tank of a vacuum filter, where Norit is added, together with sufficient lime or sulphuric acid to produce exact combination of the calcium and sulphate. Chemical agents may be added to precipitate any heavy metals still present. The mix is filtered, and the filter cake washed and disposed of. The filtered lactic acid is pumped to glass-lined tanks and adjusted to the desired concentration. Lactic acids of 50 and 44 per cent concentrations are in popular demand. The acid is placed in wooden barrels for distribution.

LACTIC ACID OF EDIBLE GRADE.—The starting material for the manufacture of the edible grade of lactic acid is the washed, solid, crude calcium lactate that is obtained from the centrifuge after the first crystallization (Fig. 34). This crude calcium lactate is added to a small amount of hot water in a wooden tank to form a solution. Sufficient sulphuric acid is added to combine all the calcium and sulphate as calcium sulphate. Finally decolorizing carbon is added, and the mix is agitated thoroughly. The precipitates are removed as a sludge by vacuum filtration. The lactic acid is stored in wooden tanks and eventually concentrated to 50 to 60 per cent strength in stainless-steel evaporating pans.

The purification treatment is repeated. Calcium and sulphate are exactly balanced, and a decolorizing carbon and a reagent to precipitate heavy metals are added. The mix is vacuum filtered, and the cake is washed. In glass-lined tanks, the lactic acid of approximately 18°Bé. is diluted to 50 or 44 per cent strength and placed in wooden barrels. Such acid is water clear.

WATER-WHITE LACTIC ACID.—This grade is of very high purity and is used by the chemical industry. Although either dry or wet U.S.P. calcium lactate may be used for its manufacture, the wet product is most generally used, since the necessity for drying is eliminated.

The purification process is similar to that employed in the making of edible-grade lactic acid. The calcium is exactly balanced with sulphate, decolorizing carbon, etc., are added, and the mix is vacuum filtered. The acid from the vacuum evaporating pan is of 18°Bé. strength or contains about 65 per cent of lactic acid.

This acid must be entirely freed of calcium. An excess of sulphuric acid is used to precipitate the last traces of this substance. The filtered acid should give no precipitate when tested with ammonium oxalate.

Barium hydroxide is added to remove the excess of sulphuric acid, although a very slight excess of sulphuric acid is desired in view of the fact that the water used for diluting the lactic acid may contain a slight amount of calcium. Thus the very slight excess of sulphuric acid will balance the calcium added by the dilution water. A final filtration removes all precipitates of barium sulphate and calcium sulphate. Should distilled water be used for diluting the water-white acid to its final strength, dilutions may be made after the final filtration.

The lactic acid is placed in barrels for marketing.

THE PRODUCTION OF WHITE CALCIUM LACTATE

A process for producing a white calcium lactate has been developed by Daly, Walsh, and Needle.[1] The special features of this process are the use of a nondenatured milk as the nutrient and the rapid drying of the calcium lactate produced.

Whole milk, buttermilk, or skim milk, in liquid or in dried form,[2] may be used as the nutrient, but the protein must not be denatured. Nondenatured milk favors a rapid fermentation, thus avoiding the formation of undesirable colors or end products, and imparts no color to the medium.

The rapid drying of calcium lactate prevents caramelization and other color changes.

[1] U.S. Patent 2,143,359, Jan. 10, 1939.

Calcium lactate may be produced according to the following example. A typical mash may contain the following ingredients:

	Pounds
Sugar (dextrose)	6,600
Milk powder	150
Diammonium acid phosphate	50
Calcium carbonate	4,600
Water to 7,000 gal.	

The foregoing mash is inoculated with 300 gal. of a 24-hr. culture of *L. delbrueckii* and incubated at a temperature of 49°C. (120°F.) for 5 to 7 days, at the end of which time the total reducing sugars are usually less than 0.1 per cent. The use of the high temperature favors *L. delbrueckii* and inhibits the development of many other types of microorganisms.

The fermented mash is heated to 82°C. (180°F.) and held at that temperature for sufficient time to destroy the microorganisms present. Enough lime is then added (100 to 200 lb.) to bring the pH of the mash to approximately 11. The mash is filtered hot, proteins and insoluble compounds being removed. The filtrate is adjusted to a pH of 6 to 7 with lactic acid, for this pH range favors the volatilization of undesired organic acids and tends to prevent the formation of undesired color during the subsequent treatment. Using a vegetable carbon, the calcium lactate liquor is decolorized to yield a solution of water-white color. The solution is concentrated quickly to 20 to 21°Bé. by exposing a shallow layer to a large heating surface. This partially concentrated liquor is next spray dried in a chamber into which gases enter at a temperature of approximately 232°C. (450°F.) and leave at a lower temperature. (Other types of drying may be substituted, if the drying is effected rapidly to prevent discoloration.) The resultant powder, maintained at a low temperature in order to avoid discoloration, is conveyed to a cyclone where it may be separated into relatively fine and coarse particles.

The product obtained by the forgoing process is noncrystalline, glass-like and homogeneous, with a calcium lactate content, based on the total solids, of 98 to 99 per cent. The moisture content is 5 to 6 per cent; the protein usually about 0.5 per cent.

If lactic acid is desired instead of calcium lactate, the filtrate from the lime treatment is decolorized with vegetable carbon and filtered. A measured quantity of sulphuric acid is then added to the filtrate, and the precipitate of calcium sulphate is removed by filtration. The resultant filtrate is decolorized with vegetable carbon and then concentrated to the desired strength by evaporation.[1]

THE PRODUCTION OF DEXTROLACTIC ACID

Tatum and Peterson[1] have described a method for producing *d*-lactic acid on a small scale. Batches containing 18 liters of a medium consisting of 3 per cent cerelose and 3 per cent malt sprouts were sterilized in 20-liter Pyrex bottles. The medium was inoculated with a pure culture of an organism that produced *d*-lactic acid. The inoculated medium was incubated at the temperature most favorable for the organism used, 30 or 37°C. After the fermentation had been in progress for 24 hr., an excess of sterile calcium carbonate was added. Each bottle was shaken frequently during the 6 to 10 days of incubation in order to neutralize the acid as it was formed. Samples of the acid were converted to zinc lactate for analyses. Table 58 gives data for some of the fermentations carried out by Tatum and Peterson.

TABLE 58.—LACTIC ACID PRODUCTION BY VARIOUS ORGANISMS[1]

Organism	Temperature of incubation of glucose, °C.	Lactic acid formed per 540 g. of glucose, grams	Glucose converted to lactic acid, per cent	Analysis of zinc lactate		Form of lactic acid
				Water of crystallization, per cent	Specific rotation,[2] $[\alpha]_D^{20}$	
Strept. lactis, R.....	30	495	91	12.52	−8.65	Dextro
Strept. lactis, R....	30	525	97	13.23	−8.65	Dextro
L. casei...........	30	505	93	12.80	−8.22	Dextro
L. delbrueckii, 3....	37	530	98	13.00	−8.22	Dextro
L. delbrueckii, 3....	37	520	96	12.90	−8.18	Dextro
L. delbrueckii, 3....	37	500	92	12.86	−8.60	Dextro

[1] TATUM, E. L., and W. H. PETERSON: *Ind. Eng. Chem.*, **27**: 1493 (1935).
[2] 4 per cent concentration.

THE PURIFICATION OF LACTIC ACID

In the purification of lactic acid, the object is to remove the ingredients that constitute impurities. These may include unfermented sugar, nitrogenous substances, coloring matter, calcium or sodium sulphate, and other salts.

Several methods may be used in purifying lactic acid. Smith and Claborn[2] of the Bureau of Dairy Industry, U.S. Department of Agriculture, have briefly discussed six methods for accomplishing purification. One method is purification by the process of recrystallizing calcium lactate, followed by treatment with sulphuric acid to liberate lactic acid.

[1] TATUM, E. L., and W. H. PETERSON, *Ind. Eng. Chem.*, **27**: 1493 (1935).
[2] SMITH, L. T., and H. V. CLABORN, *Ind. Eng. Chem.* (*News Ed.*), **17**: 641 (1939).

This process was described in detail in a foregoing section that dealt with the production of lactic acid from whey. Olive[1] and Burton[2] have described this process.

A second method consists in converting calcium lactate to zinc lactate, which crystallizes more readily than other lactates.[3] The zinc lactate is purified by repeated crystallizations. By adding hydrogen sulphide to zinc lactate, lactic acid is liberated and zinc sulphide precipitated. Animal charcoal is added to decolorize the mass, which is then filtered. The filtrate, which contains the lactic acid, is concentrated by evaporation *in vacuo*.

In another method, esters of lactic acid are prepared, purified, and subsequently hydrolyzed to liberate lactic acid in a pure form. Methanol (CH_3OH) is added to the lactate in the proportion of 10 to 20 mols of methanol to 1 mol of calcium lactate or 2 mols of sodium lactate, which, preferably, should be free from moisture. Any undissolved precipitates are removed by filtration. Sulphuric acid is added, which accomplishes two objectives, liberation of lactic acid and catalysis of the subsequent esterification. In order to esterify the lactic acid, the mixture is heated for 4 to 8 hr. at a refluxing temperature. The precipitated substances are removed by filtration, and the excess methanol by distillation at atmospheric pressure. Using a low temperature, the water and most of the methyl lactate are distilled under vacuum. After diluting the distillate in the proportion of 2 to 3 parts of distilled water to 1 part of distillate, it is slowly fractionated in a corrosion-resistant column at atmospheric pressure. After hydrolysis of the methyl lactate, the methanol is recovered and the liberated lactic acid concentrated by evaporation *in vacuo*. Smith and Claborn state that chemically pure lactic acid may be prepared most efficiently and economically by this method.

Lactic acid may be partially purified in another process by gently oxidizing the crude liquor, which contains the lactates or lactic acid. Various oxidizing agents have been used for this purpose: sodium or calcium hypochlorite, potassium permanganate, potassium chromate, nitric acid, hydrogen peroxide, chlorine gas, and ozone gas.[4]

Lactic acid may be extracted from its water solution by the use of various solvents, one of which is isopropyl ether. This process is said to be expensive and hazardous, on account of the inflammability of the ether and possibility of explosive peroxides being formed.

Fractional distillation has not been used successfully on a commercial scale.

[1] OLIVE, *loc. cit.*
[2] BURTON, *loc. cit.*
[3] SMITH, L. T., and H. V. CLABORN, *Ind. Eng. Chem.* (*News Ed.*) **17**: 641 (1939).
[4] SMITH and CLABORN, *loc. cit.*

USES OF LACTIC ACID

Lactic acid has many uses.[1,2] These include uses in connection with foods, fermentations, pharmaceuticals, and the chemical industries. As an acidulant, the edible grade of lactic acid is used in confectionery, extracts, fruit juices and essences, lemonades, pickles, sirups, and in other products. Lactic acid may be used in the curing of meat and in canned vegetable and fish products. It acts as a preservative and prevents putrefactive changes from taking place in sauerkraut and pickles. It is used to acidulate worts in the manufacture of beer, to adjust the pH of the brine in the manufacture of pickled green olives, and to inhibit the development of butyric acid bacteria in the manufacture of yeast. It is used in making sherbets and effervescent beverages.

In the chemical industries, lactic acid is used in the dyeing of silks and other textile goods, as a mordant in the printing of woolens, in the bating and plumping of leathers,[1] in the deliming of hides, in vegetable tanning, and as a flux for soft solders.[2] The water-white grade is used in the plastic industry.

The lactates also have important uses. Calcium lactate is used in baking powders, in bread, in the pharmaceutical trade and for other purposes. Iron lactate is used in pharmaceutical manufacture. Sodium lactate is employed to aid in the retention of moisture by such products as tobacco. Copper lactate is a very important agent in a new process for the electrolytic deposition of metals.

Uses have been found for derivatives of lactic acid. Ethers of lactic acid, which may be represented by the general formula $CH_3 \cdot CHOR \cdot COOH$ and which are insoluble in water, may be used as solvents, plasticizers, and modifiers[3] in the manufacture of inks, plastics, and lacquers. Esters of lactic acid, which have the general formula $CH_3 \cdot CHOH \cdot COOR$, may be used for many of the same purposes as the ethers. The higher esters, for example, the butyl, amyl, and lauryl esters, possess greater stability than the lower esters, are insoluble in water, and are therefore more desirable than the latter.

MECHANISM OF THE LACTIC ACID FERMENTATIONS

Homofermentative Bacteria.—It has been suggested that the initial stages in the lactic acid fermentation may be similar to those of the ethyl alcohol fermentation. Phosphates added to a mash accelerate the fermentation. The removal of coenzyme from lactic acid bacteria will retard or cause the fermentation to cease. *L. delbrueckii* has the ability

[1] SMITH and CLABORN, *op. cit.*, **17**: 370 (1939).
[2] BURTON, *op. cit.*, **9**: 634 (1937).
[3] SMITH and CLABORN, *loc. cit.*

to convert hexosediphosphate to methylglyoxal, likewise, to convert methylglyoxal quantitatively to racemic lactic acid.[1]

Lactic acid may arise thus through methylglyoxal by a mechanism similar to that of the alcoholic fermentation (refer to Chap. IV).

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \quad \underset{\text{Methylglyoxal}}{2CH_3COCHO} + 2H_2O \quad \text{(By mechanism similar to that of the alcoholic fermentation)}$$

$$\underset{\text{Methylglyoxal}}{CH_3{\cdot}CO{\cdot}CHO} \xrightarrow{\text{Glyoxalase}} \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH}$$

The total changes may be expressed by the following equation:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Lactic acid}}{2CH_3{\cdot}CHOH{\cdot}COOH}$$

Heterofermentative Bacteria.—Some of the first researches concerning the mechanism of the formation of the final products by heterofermentative lactic acid bacteria were carried out by Gayon and Dubourg;[2] by Fred, Peterson, and Davenport;[3] by Peterson and Fred;[4] and by others.

Nelson and Werkman[5] carried out experiments with several heterofermentative bacteria and obtained data upon which they have based a

TABLE 59.—ANAEROBIC DISSIMILATION OF GLUCOSE BY *L. acidophil-aerogenes* AND *L. lycopersici*[1]

Products	*L. acidophil-aerogenes*, millimols per liter	*L. lycopersici*, millimols per liter			
		I	II	III	IV
Glucose fermented.		80.0	92.1	102.2	112.2
Ethyl alcohol....	39.5	63.0	69.6	74.1	78.9
Acetic acid......	10.2	12.1	16.5	15.5	19.5
Carbon dioxide..	43.9	64.3	80.0	81.0	95.1
Lactic acid......	108.2	65.2	67.6	83.1	89.7
Glycerol.........	21.7	25.0	36.2	32.6	43.5
Succinic acid....	0.8	3.8			
Percentage of carbon recovered:					
With succinic acid.......	100.2	104.2			
Without succinic acid....	100.0	101.0	101.9	99.0	103.0
Oxidation-reduction ratio...	0.873	0.851	0.912	0.884	0.943

[1] NELSON, M. E., and C. H. WERKMAN, *Jour. Bact.*, **30**: 547–557 (1935).

[1] ANDERSON, C. G., "An Introduction to Bacteriological Chemistry," William Wood & Company, The Williams & Wilkins Company, Baltimore, 1938.

[2] GAYON, U., et E. DUBOURG, *Ann. Inst. Pasteur*, **15**: 527–569 (1901).

[3] FRED, E. B., W. H. PETERSON, and A. DAVENPORT, *Jour. Biol. Chem.*, **42**: 175–189 (1920).

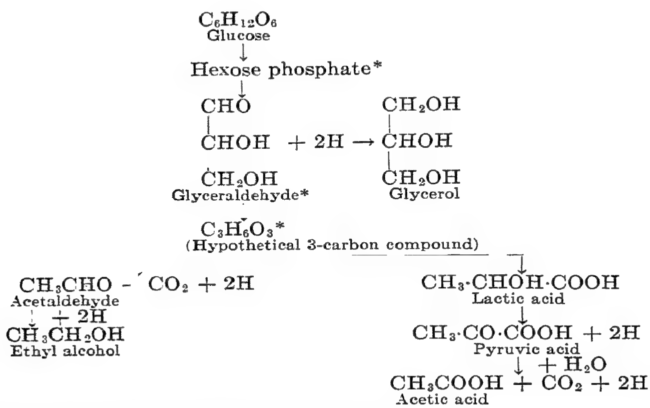
[4] PETERSON, W. H., and E. B. FRED, *Jour. Biol. Chem.*, **44**: 29–45 (1920).

[5] NELSON, M. E., and C. H. WERKMAN, *Jour. Bact.*, **30**: 547–557 (1935).

scheme for the dissimilation of glucose. The medium used by these men contained 2 per cent glucose, 1 per cent peptone, 0.3 per cent yeast extract (Difco), 0.6 per cent K_2HPO_4, and 0.6 per cent KH_2PO_4, with a pH of 6.2. Of these constituents, (1) the glucose, (2) the peptone and yeast extract, and (3) the phosphates were sterilized separately for 20 min. under a steam pressure of 20 lb. The separately sterilized components were combined just before inoculation. The mashes were incubated under an atmosphere of oxygen-free nitrogen at 30°C. for a period of 3 weeks before being analyzed.

The data of Table 59 illustrate the kinds and quantities of the products formed by *L. acidophil-aerogenes* and *L. lycopersici* from glucose under the experimental conditions just mentioned. (*L. acidophil-aerogenes* and *L. lycopersici* are listed as probable synonyms of *L. brevis* in the fifth edition of "Bergey's Manual of Determinative Bacteriology.")

Nelson and Werkman have suggested a tentative scheme for the dissimilation of glucose by heterofermentative bacteria. This scheme is given below.



* These products have not been identified in the fermentation.

In the foregoing scheme, acetaldehyde and pyruvic acid are important intermediates. When acetaldehyde, or acetylmethylcarbinol, is added to a glucose fermentation, there is an increase in the quantities of acetic acid and carbon dioxide, but a decrease in the quantities of ethanol, lactic acid, and glycerol formed from glucose.[1] Since both acetaldehyde and acetylmethylcarbinol are hydrogen acceptors, they will compete with the hydrogen acceptors, such as glyceraldehyde, which arise normally during the fermentation. The result will be a decrease in the

[1] *Ibid.*, **31**: 603–610 (1936).

quantities of the compounds that are usually formed by reduction from these intermediary hydrogen acceptors.

The acetaldehyde added to a fermenting medium is reduced to ethanol. Nelson and Werkman[1] have shown that pyruvic acid may be fermented by *L. lycopersici* with the production of equimolar amounts of acetic, lactic, and carbonic acids:

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + H_2O \rightarrow \underset{\text{Hydrated pyruvic acid}}{CH_3{\cdot}\overset{OH}{\underset{OH}{\dot{C}}}{\cdot}COOH} \rightarrow \underset{\text{Acetic acid}}{CH_3COOH} + CO_2 + 2H$$

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + 2H \rightarrow \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH}$$

One molecule of pyruvic acid is hydrated to form 1 molecule each of acetic acid and carbon dioxide (carbonic acid), while a second molecule is reduced to a molecule of lactic acid.

Mannitol Fermentation.—The fermentation, by lactic acid bacteria, in which mannitol is one of the products, has been studied by Bolcato[2] and by Schoen and Eras.[3] The mechanism of this fermentation will not be discussed here. For details in connection with this and the other types of fermentations discussed in the preceding paragraphs, the interested reader is urged to consult the references cited.

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[2] BOLCATO, V., *Ann. chim. applicata*, **26**: 24, 356, 423 (1936); *Enzymologia*, **5**: 52 (1938).

[3] SCHOEN, M., and E. ERAS, *Enzymologia*, **4**: 198 (1937).

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CHAPTER XVII

SAUERKRAUT

Sauerkraut is the clean, sound

. . . product of characteristic flavors, obtained by full fermentation, chiefly lactic, of properly prepared and shredded cabbage in the presence of not less than 2 per cent, nor more than 3 per cent salt. It contains, upon completion of the fermentation, not less than 1.5 per cent of acid, expressed as lactic acid. Sauerkraut which has been rebrined in the process of canning or repacking contains not less than 1 per cent of acid, expressed as lactic acid.[1]

The fermentation is a natural one induced by bacteria resident on the leaves of the cabbage. By suitable control procedures involving selection and cleanliness of materials and regulation of the conditions of fermentation, an excellent food product is made.

Varieties of Cabbage Used for Sauerkraut Manufacture.—Sauerkraut may be manufactured from many different varieties of cabbage, but the varieties that grow slowly and form closely filled heads are preferred. All Seasons, All Head Early, Glory of Enkhuizen, and Flat Dutch are some of the desirable varieties.[2]

The variety grown will depend in part on local conditions of the climate and soil available.

Fully matured cabbage should be used for sauerkraut manufacture, since the use of green cabbage often leads to the production of kraut that is defective in color and in texture.[3]

Composition of Cabbage.—Various analyses have been given for cabbage.[4] Atwater and Bryant[5] state that cabbage contains 86 to 94.3 per cent water, 2.9 to 6.4 per cent sugar, 0.2 to 2.4 per cent protein, 0.1 to 0.7 per cent fat, 0.5 to 1.6 per cent fiber, and 0.4 to 2.4 per cent ash. Peterson, Fred, and Viljoen,[6] in analyses of fresh cabbage, found 91.0 to 93.9 per cent water, 3 to 4.2 per cent sugar, and 0.15 to 0.24 per cent total nitrogen.

[1] U.S. Dept. of Agriculture, F.D.A., Service and Regulatory Announcements, Food and Drug, No. 2, Rev. 5, November, 1936.

[2] LeFevre, E., *U.S. Dept. Agr., Circ.* 35, June, 1928.

[3] *Ibid.*

[4] Winton, A. L., and K. B. Winton, "The Structure and Composition of Foods," Vol. II, John Wiley & Sons, Inc., New York, 1935.

[5] Atwater, W. O., and A. P. Bryant, *U.S. Dept. Agr., Bull.* 28, 1906.

[6] Peterson, W. H., E. B. Fred, and J. A. Viljoen, *Canner*, **61**: 19 (1925).

Commercial Manufacturing Procedure.—Preparatory to shredding, sound cabbage is stored in well-ventilated buildings for at least 1 day to induce wilting and to obtain a product of more uniform temperature. The wilted cabbage should be cut with the production of the least amount of injury to the shreds, for damage might result in a subsequent softening of the sauerkraut. The spiral drill of a coring device cuts the core but does not remove it from the cabbage, which is passed on a conveyor to a trimming table where the outer leaves and any undesirable portions are removed. The cabbage is sliced by machine and the shreds are transported to the fermentation vats, some of which have a capacity of at least 80 tons of sauerkraut. Salt may be applied evenly to the shredded cabbage as it is being distributed in the vat or it may be spread through the product in the carts that carry the cabbage from the cutting rooms to the vats. A round cover is placed over the salted cabbage in each vat in such a manner that very little of the cabbage is left exposed to the air. Weights, usually cement blocks with handles, are placed on the cover to force the cabbage under the juices extracted from the shredded and salted product. When the acid content has reached at least 1.5 per cent, a process that may require from 3 to 4 or more weeks, depending on the temperature, the fermentation is usually complete.

Products of Fermentation.—Juices containing the sugars and other soluble substances are withdrawn from the shredded cabbage by the action of the salt. The bacteria that bring about the characteristic sauerkraut fermentation act upon the sugars and break them down to lactic and acetic acids, mannitol, ethyl alcohol, carbon dioxide, and small amounts of other products. Esters are formed from alcohol and the organic acids.

Microorganisms.—Many types of microorganisms may be found on the cabbage. Pederson[1] of the New York State Agricultural Experiment Station classifies the more important microorganisms that may affect fermentation into three groups: one group contains those bacteria which bring about the normal sauerkraut fermentation; one group contains spoilage bacteria; and one group contains yeasts.

Desirable Types.—The common lactic-acid-producing bacteria that produce a normal sauerkraut fermentation may be further divided into three types: gas-producing cocci, non-gas-producing rods, and gas-producing rods. These three types are the only ones to develop in the normal controlled fermentation.[2] During the first part of the sauerkraut fermentation, the gas-producing cocci, *Leuconostoc mesenteroides*, predominate, for they find the salt concentration of 2.5 per cent and the temperature of 70°F., or below, favorable. These cocci attack the sugars

[1] PEDERSON, C. S., *N.Y. State Agr. Expt. Sta., Bull.* 595, 1931.

[2] PEDERSON, C. S., *N.Y. State Agr. Expt. Sta., Tech. Bull.* 168, 1930.

incompletely and produce lactic and acetic acids, ethanol, mannitol, and carbon dioxide. They produce the least amount of change in the protein and in the cellular structure of the sauerkraut. When the acid content increases to 0.7 to 1.0 per cent, they are destroyed. During their development, esters, which impart flavor to the sauerkraut, are formed from the acids and alcohol produced by them.

The two other types of lactic-acid-producing bacteria do not develop rapidly at first under the conditions of the fermentation, but they gradually gain ascendency and carry the fermentation to completion. One of these types, the non-gas-producing rods, includes *Lactobacillus cucumeris* (a probable synonym of *L. plantarum*) and *L. plantarum,* which produce lactic acid in large quantities (refer to the section on homofermentative lactobacilli) from a portion of the unchanged sugar and the mannitol, the latter substance having been produced from sugar by the gas-producing cocci. These lactobacilli are slowly destroyed, owing to their lack of resistance to the acid produced.

Fermentation is completed by the third type, *L. pentoaceticus* (a probable synonym of *L. brevis*), which produces lactic and acetic acids, ethanol, mannitol, and carbon dioxide from the remaining sugars. This organism is quite resistant to acid, withstanding an acid content as high as 2.4 per cent.

The following table, from Pederson's researches, shows analyses of partially and completely fermented sauerkrauts.

TABLE 60.—ANALYSES OF PARTIALLY AND COMPLETELY FERMENTED KRAUTS[1]

Kraut no.	Total acid, per cent	Lactic acid, per cent	Acetic acid, per cent	Acetic acid/ lactic acid ratio	Alcohol, per cent	Mannitol, per cent	Sugars
Partially Fermented Kraut							
108	0.85	0.535	0.210	0.39	0.44	0.58	Trace
109	0.98	0.615	0.237	0.38	0.32	0.54	Trace
111	0.94	0.515	0.283	0.55	0.36	0.42	Trace
113	0.88	0.530	0.234	0.44	0.32	0.39	Trace
Completely Fermented Kraut							
109	1.67	1.268	0.268	0.21	0.62	Trace	None
111	1.78	1.304	0.318	0.24	0.47	Trace	None
113	1.67	1.183	0.324	0.27	0.65	Trace	None

[1] PEDERSON, C. S., *N.Y. State Agr. Expt. Sta., Bull.* 595, 1931.

A marked increase in the quantity of lactic acid with but slight increases in the acetic acid and alcohol content indicate that the non-

gas-producing lactobacilli have dominated during the last stages of the fermentation.

The spoilage types of bacteria are usually aerobic and attack the proteins, exert but little action toward the sugars, and fail to grow in acid media. In the normal fermentation this group fails to gain ascendency.

Yeasts grow in the presence of oxygen, attack the sugars and lactic acid, but rarely develop during the early part of the fermentation. They may cause pink sauerkraut unless controlled.

Starters.—The use of starters is not advocated, although under certain circumstances good to excellent results have been secured through their use.

A starter may consist of a pure culture, such as *Leuconostoc mesenteroides* or *Streptococcus lactis;* of a mixed culture, containing those organisms normally found in the sauerkraut fermentation; or of sauerkraut juice.

Starters containing pure cultures of the cocci that predominate during the initial period of the fermentation, or kraut juice, with an acidity of 0.25 per cent or less, withdrawn during the early stage of a fermentation, may bring about a normal fermentation. The value of the use of such starters is questionable, according to Pederson.[1]

When the sauerkraut juice contains more than 0.30 per cent of acid, the development of *L. mesenteroides* is inhibited and the resultant kraut is of an inferior quality. The non-gas-producing rods predominate under these conditions.

Old sauerkraut juices, juices containing more than 0.30 per cent acid, and cultures in which the lactobacilli (*Lactobacillus cucumeris* and *L. pentoaceticus*) predominate usually produce sauerkrauts of a poorer quality. For these reasons, sauerkraut manufacturers are advised to wash thoroughly and steam any vats in which a sauerkraut fermentation is contemplated and in which a fermentation has just been completed.

Three factors are of much importance in regulating the fermentation of cabbage: the concentration of the salt, the covering of the sauerkraut, and the temperature of fermentation.

Salt.—Salt serves several important functions in the sauerkraut fermentation. It draws the juices out of the cabbage; it favors a lactic acid fermentation, the acid of which checks putrefaction; it contributes to the flavor; and it has much to do with firmness of the final product.

The greater the amount of salt used, the more effective will be the extraction of juices, and the firmer will be the shredded cabbage leaves. But high concentrations of salt will inhibit the desirable fermenting bacteria to some extent. It becomes necessary, therefore, to select a concentration that will permit a normal fermentation and give a firm,

[1] PEDERSON, C. S., *N.Y. State Agr. Expt. Sta., Tech. Bull.* 216, 1933.

high-grade sauerkraut. A salt concentration of 2.5 per cent is favored by many commercial sauerkraut manufacturers—2.5 lb. of salt per 100 lb. of cabbage.

Spoilage types are not inhibited to any great extent until a salt content of 5 to 7 per cent is used. A high concentration of salt, therefore, cannot be depended on to prevent spoilage. This concentration of salt is, moreover, prohibitive for the sauerkraut fermentation.

The salts used in the fermentation should be of a known, pure grade free from calcium and magnesium salts and should be measured by weight rather than by volume, since densities vary for different salts. Salt should be thoroughly mixed with the shredded cabbage.

In 1916, Henneberg[1] advanced the opinion that salt is added to cabbage only to withdraw juice. Hof[2] (1935) concluded, as the result of experiments in which 2.5 per cent salt was added to cabbage in one case and water but no salt in the second case, that the most important reason for adding salt to cabbage was to promote the formation of juice. The sauerkraut made without salt was much softer than that prepared with salt. Gerson[3] states that sauerkraut may be made by adding water, without salt, to the cabbage. Such sauerkraut is very soft, however.

The salt concentration of a sauerkraut may be determined by titrating a sample of the juice with 0.1 N silver nitrate, using potassium chromate as the indicator.

Covering the Sauerkraut.—The covering of sauerkraut during fermentation is necessary to exclude oxygen, which favors the development of yeasts and spoilage types of bacteria. (See the section on sauerkraut spoilage.) Weights are placed on the cover in a vat to force the extracted juices over the uppermost layer of sauerkraut. Muslin cloth is sometimes used to aid in maintaining moisture in the product at the top of the vat.

Temperature.—The optimum temperature for the sauerkraut fermentation is believed to be 65 to 70°F. (18.3 to 21.1°C.), while the range of 60 to 70°F. (15.6 to 21.1°C.)[4] is satisfactory. Any temperatures below 60°F. (15.6°C.) retard the fermentation. As was stated earlier, *Leuconostoc mesenteroides* grows well at temperatures below 70°F. (21.1°C.) but at 60°F. (15.6°C.) or below, the fermentation will usually cease after the production of about 1 per cent of acid, since the lactobacilli fail to develop.

Experience has shown that the use of high temperatures for the fermentation is also undesirable. The use of temperatures around 86°F. (30°C.) favors the development of the lactobacilli but retards the develop-

[1] Henneberg, W., *Deut. Essigind.*, **20**, No. 21–22 (1916).

[2] Hof, T., *Rec. trav. botan. néerland.*, **32**: 93 (1935).

[3] Gerson, M., "Meine Diät," p. 96, Ullstein, Berlin, 1930.

[4] Pederson, C. S., *N.Y. State Agr. Expt. Sta., Bull.* 595, 1931.

Parmele, H. B., E. B. Fred, W. H. Peterson, J. E. McConkie, and W. E. Vaughn, *Jour. Agr. Research*, **35**: 1021 (1927).

ment of *Leuconostoc*, which is primarily a flavor-producing organism. In order to maintain cabbage at this temperature, artificial heating must be employed. If the building is heated to above 86°F. (30°C.), as would be necessary, spoilage is likely to be incurred.

Spoilage of Sauerkraut.—Aside from purely aesthetic reasons the importance of maintaining a sauerkraut factory in a high state of cleanliness is emphasized by a study of the following causes of spoilage.

Dark sauerkraut is not uncommon and may be caused by a number of factors. Exposure of the sauerkraut to air may produce a dark product. Too much salt in certain cases, the result of uneven distribution, causes darkening of sauerkraut ("burned" sauerkraut), since normal fermenters but not spoilage types of bacteria are inhibited by high salt concentrations. Darkened sauerkraut may be induced by the use of excessively high temperatures during fermentation, conditions that favor the development of abnormal types of bacteria. The presence of iron and tannin (from wood), or the surface rotting of sauerkraut may also produce a darkened product. When the fermenting product is improperly weighted and the juice fails to cover or moisten the surface, aerobic bacteria and yeasts are likely to produce a darkening of the sauerkraut at the surface owing to the faulty fermentation that they cause.

Pink sauerkraut is caused by certain kinds of yeast. The types found on sauerkraut develop only when air is present. Hence they are usually found in the juice at the surface of the sauerkraut, occasionally as a white scum, when not well covered, or in the air pockets formed between the sides of the vat and the shredded cabbage, especially when the salt and cabbage have been unevenly mixed. Sugar and, in some cases, acid may be attacked by them. They also produce a pigment that may vary from a pink to an intense red in color. In completely and properly fermented normal sauerkraut these organisms do not usually develop.

The growth of pink yeasts is favored by factors that inhibit the normal sequence of bacterial growth.[1] Thus a high temperature of fermentation, a salt concentration greater than normal, an increased acid content, and a cabbage of low nitrogen content[2] may favor the growth of the yeasts producing a pink or red color in sauerkraut.

Soft sauerkraut may be due to the use of too little salt, high temperatures, faulty fermentation, or exposure to the air. The use of insufficient salt may produce a soft sauerkraut because it fails to draw enough juice from the cabbage or because it permits the lactic-acid-producing rods to develop. A high temperature favors the growth of lactobacilli at the beginning of the fermentation rather than in their proper sequence, with

[1] PEDERSON, C. S., and C. D. KELLY, *Food Research*, **3**: 583 (1938).
[2] PETERSON, W. H., H. B. PARMELE, and E. B. FRED. *Soil Sci.*. **24**: 299 (1927).

the result that the structure of the cabbage leaf is broken down to some extent. In such cases, the cocci fail to develop properly. Improper cleaning of a vat that has been packed previously, combined with high temperatures, favors the predominance of those types of bacteria which are commonly found at the end of the fermentation. These bacteria have, as previously stated, a greater ability to weaken the structure of the cabbage. Softening of sauerkraut by bacteria or yeasts growing on the surface of a product that has undergone a normal fermentation is not common.

Slimy sauerkraut is caused usually by the rapid growth of certain strains of *Lactobacillus cucumeris* and *L. plantarum,* especially at raised temperatures. Such sauerkraut is edible but of an objectionable character. The slime may sometimes be dissolved by canning or cooking.

Rotted sauerkraut may be caused by molds, yeasts, bacteria, or, once in a while, fruit flies. A heavy muslin cloth placed over the surface of the shredded cabbage and pushed down the sides of the vat at the time of packing it may be of considerable value in preventing rot. The cover is placed directly over the cloth with enough weights on top of it to press down the cabbage without causing the juice to flow over the cloth. Since the surface of the fermenting sauerkraut is kept moist the growth of undesirable types of bacteria will be inhibited. Flies will be excluded by the cloth.

Off flavors may result from changes in the normal sequence of bacterial development, or from too rapid a fermentation.

Cabbage for Sauerkraut.—Table 61 gives information concerning the production of cabbage for sauerkraut.

An examination of this table indicates that New York produces more than one-third of all the cabbage used for sauerkraut manufacture, while Wisconsin produces more than one-fifth. Approximately three-fifths of the total quantity of cabbage is thus produced by New York and Wisconsin.

Ohio is the third largest producer of cabbage for sauerkraut manufacture.

Growers receive different prices per ton for the cabbage used for sauerkraut manufacture. In 1928, the average price received by growers was $9.54 per ton;[1] in 1930, $7.74; in 1932, $4.11; in 1935, $5.17; and in 1936, $13.06.

Prices paid in different states varied considerably; for example, the average price received by growers in Ohio was $6.80 per ton in 1936, while that for growers in Colorado was $30.00 per ton.[1]

A large quantity of sauerkraut is canned annually.

[1] U.S. Dept. of Agriculture, Bureau of Agricultural Economics, December, 1936.

Other information of interest concerning the statistics of cabbage production for sauerkraut manufacture and the location of plants will be found in *Technical Bulletin* 646, published by the U.S. Department of Agriculture.[1]

TABLE 61.—CABBAGE FOR KRAUT: 1938 WITH COMPARISONS[1]

State	Acres			Yield per acre, tons (2,000 lb.)			Production, tons		
	10-year average, 1927–1936	1937	1938	10-year average, 1927–1936	1937	1938	10-year average, 1927–1936	1937	1938
New York	6,280	8,600	5,270	9.7	6.3	15.2	58,700	54,200	80,100
Ohio	2,220	1,800	2,430	8.5	5.7	8.0	18,900	10,300	19,400
Indiana	1,410	1,350	1,100	6.5	6.4	8.3	8,600	8,600	9,100
Illinois	670	590	330	6.6	6.5	8.2	4,200	3,800	2,700
Michigan	1,310	1,400	1,100	7.5	5.2	10.4	9,800	7,300	11,400
Wisconsin	4,640	6,700	4,550	7.4	5.3	10.0	34,500	35,500	45,500
Minnesota	330	210	200	7.7	4.3	6.9	2,700	900	1,400
Colorado	330	340	230	10.8	9.9	13.1	3,600	3,400	3,000
Washington	310	300	300	8.8	8.0	8.0	2,700	2,400	2,400
Other states[2]	1,660	3,550	1,740	7.0	6.3	9.9	11,000	22,500	17,200
Total, all states	19,160	24,840	17,250	8.22	5.99	11.14	154,700	148,900	192,200

[1] U.S. Dept. of Agriculture, Bureau of Agricultural Economics, December, 1938.
[2] "Other states" includes: Iowa, Maryland, New Jersey, North Carolina, Oregon, Pennsylvania, Tennessee, Texas, Utah and Virginia.

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CHAPTER XVIII

PICKLES

Pickles are

> . . . . immature cucumbers properly prepared, without taking up any metallic compounds other than salt, and preserved in any kind of vinegar, with or without spices. Pickled onions, pickled beets, pickled beans, and other pickled vegetables are vegetables, prepared as described above, and conform in name to the vegetables used.[1]

Although almost any cucumbers, free from disease, may be used to make pickles, it is customary to select certain varieties, such as Snow's Perfection, Chicago Pickling, or Boston Pickling, which are preferable on account of their size, shape, or keeping qualities.[2]

Salt pickles, "immature cucumbers preserved in a solution of common salt, with or without spices,"[1] may be converted into sour pickles, sweet pickles, mixed pickles, or other pickle products.

The cucumbers, picked while still green, are inspected, graded, washed to remove any adhering soil, weighed, placed in vats, and salted. The cucumbers should be salted, if possible, on the same day, within a few hours of collection, in order to prevent deterioration. Decomposed pickles are a source of infection to the pickling tank and should not be used under any circumstances.

Tanks are usually constructed in the open, although they may be erected in buildings. Microbiological scums are less likely to form in tanks located in the open, owing to the action of sunlight.[3] Tanks or vats are made usually of cypress, fir, pine, or redwood;[3] are 6 to 8 ft. in height and up to 16 ft. in diameter; and have capacities for 100 to 1,600, or more, bushels of cucumbers. They must be thoroughly cleaned before use and are sometimes soaked with water containing 50 p.p.m. of chlorine. The tanks, which are frequently owned by pickling concerns, are often referred to as salting stations.

Salting.—There are in general two methods for adding brine to cucumbers, although there are many variations of these methods, and although pickles may be dry salted. In one method, a low-salt curing

[1] *U.S. Dept. of Agriculture,* F.D.A., Service and Regulatory Announcements, Food and Drug, No. 2, Rev. 5, November, 1936.

[2] LeFevre, E., *U.S. Dept. Agr., Farmers' Bull.* 1438, April, 1927.

[3] Wormley, W. G., *Food Industries,* **11**: 486 (1939).

process, the cucumbers are added to a tank partially filled with brine, which gives a salinometer reading of 30° (nearly 8 per cent sodium chloride). This concentration of salt is considered to be the lowest that can be used with safety under ordinary conditions. Salt is then added in the proportion of 9 lb. for each 100 lb. of cucumbers in the tank. Thereafter sufficient salt is added to raise the brine 3° salinometer each week until a final reading of 60° (15.9 per cent) is obtained. Fermentation and curing are rapid by this method, but there is greater danger of undesirable bacteria predominating and the pickles are not likely to be so firm as when a higher initial concentration of salt is used.

In the second procedure, a brine containing 10.6 per cent salt (40° salinometer) is used. The cucumbers are placed in the brine, and salt is added in the proportion of 9 lb. per 100 lb. of cucumbers. The salt concentration of the brine is increased 2° salinometer each week until it reaches 50° and thereafter 1° each week until a final reading of 60° salinometer is obtained. Fermentation and curing by this procedure are slower, but there is less danger of spoilage types of microorganisms predominating, and the pickles are firmer than by the low-salt curing method. This procedure is especially desirable where temperatures are likely to be relatively high, as in the South. Likewise, the higher initial concentration of salt prevents freezing of the contents of the tank in cold regions.

It is essential that very hard waters should not be used for pickle brines. The salt should be of a high quality and should contain less than 1 per cent of sodium, calcium, or magnesium carbonates or bicarbonates, for these salts will neutralize the acid produced by the lactic acid-producing bacteria and favor the development of proteolytic types.

It is the practice with some picklers to add starters from actively fermenting tanks or other containers. Such starters contain acid-producing bacteria of a desirable type. These bacteria are accustomed to high concentrations of salt and under such circumstances the acidity of the brine will increase rapidly. Since cucumbers contain less sugar than sauerkraut and often possess more mixed types of microorganisms, the use of a starter is frequently highly desirable. Sugar to the extent of approximately 1 per cent may be added to favor desirable fermentation, especially if the pickles are deficient in sugar, according to LeFevre.

Once the tank has been filled, the curing and fermentation should be carefully controlled. The pickles should be kept well covered by brine at all times by the use of false covers or heads, which are fastened in place. The strength of the brine should be checked daily. After a tank has been filled and salt has been added, it is usually desirable to pump the brine from the bottom of the tank to the top in order to insure a uniform concentration of the brine. Any scum, containing yeasts or molds,

that forms on the surface of the brine should be removed periodically to prevent consumption of the acids formed by fermentation.

Influence of Salt.—Cucumbers contain about 90 per cent of water. When salt, in the form of a brine, is added to a tank containing them, water is withdrawn from them by osmosis. Dissolved in the water are sugar, soluble proteins, minerals, and other substances, which are used as food by the lactic acid bacteria and any other microorganisms present, provided the conditions are favorable.

The brine is diluted and weakened as the result of the water being withdrawn from the cucumbers. At the same time some of the salt is absorbed by the cucumbers. Under these circumstances the salt concentration of the brine may become so reduced that spoilage types of bacteria might grow and perhaps predominate unless salt were added to maintain a fairly high concentration of the brine.

Fabian and his associates[1] studying the influence of salt on bacterial activity in the cucumber fermentation found that the bacteria present during the first 24 hr. were largely peptonizers. After this period there was a gradual diminution in the numbers of bacteria until the brine reached 50° salinometer. The numbers then became fairly constant. The peptonizers were gradually replaced by acid-producing bacteria. They died out more rapidly in 40° brine than in 30° brine, although the number of acid-producing bacteria was greater and "reached a maximum" quicker in the 30° brine than in the 40° brine.

The curing and fermentation of cucumbers, a process that is influenced by the salt concentration of the brine, the temperature, the size of the cucumbers, and the control of the scums (molds, yeasts), may require 6 to 9 weeks. During the process, the flavor, texture, and color of the cucumbers change. The cucumbers are converted from a pale green to an olive or dark green in color. The color change extends throughout the pickle. Thus, if the pickle is opaque or white internally, it is not properly cured. Lactic acid bacteria are responsible for these changes, while salt is the most important factor in controlling the fermentation. Salt tends to preserve the chlorophyll and other constituents of the pickle.

Processing.—Salt pickles are processed in water to remove some of the salt contained in them. The amount of salt removed depends upon the use that is to be made of the pickles. Unless they are used as salt pickles, a large part, but not all, of the salt is removed.

The salt pickles are placed in tanks equipped with a supply of steam. The temperature is raised to 120°F. (49°C.), or above, and the pickles agitated by means of paddles or compressed air. The water may be

[1] Fabian, F. W., C. S. Bryan, and J. L. Etchells, *Mich. Agr. Expt. Sta., Tech. Bull.* 126, November, 1932.

changed and the process repeated until the desired salt content is obtained.

Grading.—Pickles are sorted in order to obtain a product that will be appealing to the consumer. They may be graded for shape and size. Straight pickles constitute the highest grades; the nubs and crooked ones, a lower grade; while broken and poorly shaped pickles may be used in the production of sweet relish or chowchow.

Pickles 2 to 3 in. in length are classified as small pickles and are used for bottling and sweet pickles. Medium-sized pickles, those 3 to 4 in. long, are used in making dill or sweet pickles, while large pickles, 4 in. or more in length, are used principally for sour pickles.

Sour pickles are made from salt pickles that have been processed, drained, and covered with vinegar. A 45- to 50-grain vinegar, distilled vinegar being preferred usually, is added to cover the pickles. When very sour pickles are desired, it is customary to add a 40- to 45-grain vinegar first and after a few days to replace it with the desired strength of vinegar, which may be as high as 60 to 75 grains.

Dill Pickles.—Genuine dill pickles possess a fine flavor that is the result of aging and of biochemical changes brought about by microorganisms (principally bacteria) during fermentation.

The method for preparing dill pickles differs from that of preparing salt pickles in two important respects: a weaker brine is used, and spices are added to the cucumbers. The spices do not interfere with the normal fermentation, but the salt concentration of the brine is low enough to permit the growth of undesirable microorganisms. Hence special precautions should be observed in the manufacture of dill pickles.

Dill pickles are usually prepared in barrels that have been carefully cleaned and sometimes paraffined. Part of the dill (green, dry, or brined) and the other spices are placed at the bottom of the barrel. Cucumbers are added and the rest of the spice distributed evenly at the middle and top of the barrel. The barrel may or may not be headed, brine is added, and the barrel incubated at a temperature of around 70°F. (21.1°C.).

The addition of sugar, 2 lb. per barrel, favors the early development of bacteria. Sufficient vinegar or acetic acid to bring the pH to 4.6 is desirable, while the addition of a starter is advocated.

One formula for dill pickles calls for the use of 1 lb. of the following mixture in a 45-gal. barrel: 4 lb. of whole allspice, 2 lb. of coriander or mustard seed, 2 lb. of black pepper, and 1 lb. of bay leaves. From 6 to 8 lb. of green or brined dill or 3 to 4 lb. of dried dill are added to the same barrel, while a 7 to 8 per cent brine is commonly used.

In the dill-pickle fermentation, Gram-positive cocci predominate[1] at the beginning. Short rods, the majority of which are Gram-positive, replace the cocci. Toward the end of the fermentation, long rods predominate, the majority of these being Gram-positive. In approximately 8 to 10 days after the start of the fermentation the strong-acid-producing bacteria are at a maximum.

Imitation dill pickles may be prepared from salt pickles that have been processed, soured, drained, and placed in tight containers with dill, other spices, vinegar, and brine. The acid of imitation dill pickles is principally acetic acid, while in genuine dill pickles the acid is mainly lactic acid.

Sweet pickles are "pickled cucumbers or other vegetables sweetened with sugar and/or dextrose."[2] They may be prepared from salt pickles that have been soured and drained. A sirup is made by dissolving 4 to 10 lb. of sugar in 1 gal. of vinegar. The pickles are placed in a container, and one-half their weight of sirup is poured over them, and spices are usually added. At the end of 2 to 4 months, the sugar is largely inverted and the pickles have become darker green in color.

Wadsworth and Fabian[3] have discussed the use of lactic acid in combination with acetic acid in the finishing of sweet pickles, processed dill pickles, and pickle products. Ratios of lactic acid to acetic acid of 1:4 (0.4 per cent of lactic acid, as acetic acid, to 1.6 per cent acetic acid), or 1:9 produced the best results.

Mixed pickles consist of two or more vegetables in the same container —cucumbers, cauliflower, green peppers, onions, beans, or green tomatoes being common vegetables used in the manufacture of mixed pickles.

Kosher dills are dill pickles containing garlic as an important spice.

Spoilage of Pickles.—*Soft pickles* are caused by the action of bacteria of the *Bacillus mesentericus* (*B. mesentericus fuscus*) group or by strong or weak acids. The insoluble protopectin of the pickle is converted to a soluble pectin by protopectinase, an enzyme, by strong acids, or by weak acids over a period of time.[4]

Hollow pickles may be due to any one of a number of factors: thickness of skin, too rapid fermentation, loose packing, insufficient weighting of the tank, or the concentration of the brine.

Shriveling is usually caused by placing the cucumber or pickle in too strong a salt, sugar, or vinegar solution.

[1] FABIAN, F. W., and L. J. WICKERHAM, *Mich. Agr. Expt. Sta., Tech. Bull.* 146, July, 1935.

[2] *U.S. Dept. of Agriculture*, F.D.A., Service and Regulatory Announcements, Food and Drug, No. 2, Rev. 5, November, 1936.

[3] WADSWORTH, C. K., and F. W. FABIAN, *Food Industries*, **11**: 252, 324 (1939).

[4] FABIAN, F. W., and E. A. JOHNSON, *Mich. Agr. Expt. Sta., Tech. Bull.* 157, February, 1938.

Pickle blackening may be caused by *B. nigrificans*, a strain of *B. mesentericus*. The presence of a carbohydrate, such as dextrose, the absence of adequate quantities of nitrogenous substances, and a reaction that is practically neutral or slightly alkaline favor blackening.[1] A water-soluble pigment produces the darkening of the brine and pickles.

The production of iron sulphide is also a cause of pickle blackening. Hydrogen sulphide (H_2S) may be produced by bacteria or by chemical means. In the presence of iron salts, the sulphide of iron is produced. Iron sulphide is formed at a neutral or slightly alkaline reaction.[2]

The salinometer (or salometer) is an instrument that is very useful in measuring the salt concentration of a brine. The scale on the salinometer is divided into 100 degrees, the zero graduation indicating the reading of the instrument when placed in pure water at 60°F. and the 100° graduation indicating the reading obtained in a saturated solution of sodium chloride at 60°F. (26.5 per cent salt). Each degree represents slightly more than one-quarter of 1 per cent (0.265 per cent) of salt. Table 62 gives a comparison of salt percentages and the corresponding salinometer readings.

Pickled Green Olives.—Briefly, the production of pickled green olives consists of selecting suitable varieties, harvesting, grading, treating with lye, washing with water, brining, and packaging. In order to obtain a high-grade product, it is necessary to use considerable care in each of the various operations.

Varieties.—Cruess[3] lists five varieties of olives that are suitable for green pickling. These include the Sevillano, Barouni, Manzanillo, Ascolano, and Mission olives, the descending order of their suitability being given.

Harvesting.—The olives should be picked when full sized but free of any pink or red color. Green to partially yellow olives are preferred. Olives that are straw-yellow to pink in color do not produce the best pickled products.

Grading.—The overripe, bruised, or blemished olives are removed and, if the olives will not be bruised in the process, they are graded, otherwise grading is deferred until after the fermentation.

Lye Treatment.—Olives are treated with a dilute lye (sodium or potassium hydroxide) to remove a portion but not all of the bitter principle, for some bitterness is desirable for flavor.

The concentration of lye used depends upon the variety of olive being treated. A 1.60 per cent lye, or one slightly less concentrated, is advo-

[1] Fabian, F. W., and A. L. Nienhuis, *Mich. Agr. Expt. Sta., Tech. Bull.* 140, August, 1934.

[2] Fabian, Bryan, and Etchells, *op. cit.*

[3] Cruess, W. V., *Calif. Agr. Expt. Sta., Bull.* 498, October, 1930.

cated by Cruess for Sevillano and Ascolano olives, but a 1.7 to 2 per cent lye is advocated for Manzanillo, Barouni, and Mission olives because they contain more of the bitter principle. The use of too concentrated lye or prolonged treatments will remove all the bitter principle and impair the flavor, color, and texture.

TABLE 62.—SALT PERCENTAGES AND CORRESPONDING SALINOMETER READINGS

Salt in solution, per cent	Salinometer reading, degrees	Salt in solution, per cent	Salinometer reading, degrees
0	0	10.07	38
0.265	1	10.6	40
0.53	2	11.13	42
0.795	3	11.66	44
1.06	4	12.19	46
1.325	5	12.72	48
1.59	6	13.25	50
1.855	7	13.78	52
2.12	8	14.31	54
2.385	9	14.84	56
2.65	10	15.37	58
2.915	11	15.9	60
3.18	12	16.43	62
3.445	13	16.96	64
3.71	14	17.49	66
3.975	15	18.02	68
4.24	16	18.55	70
4.505	17	19.08	72
4.77	18	19.61	74
5.035	19	20.14	76
5.3	20	20.67	78
5.565	21	21.2	80
5.83	22	21.73	82
6.095	23	22.26	84
6.36	24	22.79	86
6.625	25	23.32	88
6.89	26	23.85	90
7.155	27	24.38	92
7.42	28	24.91	94
7.95	30	25.44	96
8.48	32	25.97	98
9.01	34	26.5	100
9.54	36		

The time required for treating the olives with lye is dependent on the temperature of the process, the concentration of the alkali, the size of the olives, and depth of penetration desired. The temperature of the lye is generally 70 to 75°F. (21.1 to 23.9°C.). Lye is permitted to penetrate one-half way to the pit in Sevillano and Ascolano olives and

two-thirds of the way in Manzanillo, Barouni, and Mission olives. The process usually requires 4 to 6.75 hr., during which time the olives should be stirred occasionally.

The progress of lye penetration is followed by removing olives from time to time, slicing them, placing a few drops of phenolphthalein on the cut surface and observing the pink to red color that indicates the depth to which the lye has penetrated.

Washing.—The lye-treated olives are washed to remove the alkali. The olives are covered with clean water which is allowed to stand for 2 or 3 min. and then drained off. This process is repeated for perhaps three times, and thereafter the water is changed about once every 3 hr.

Ordinarily about 30 hr. may be required for washing, but the actual time will depend on the frequency of changing the water, the size of the olives, the depth of penetration of the lye in the olives, and the concentration of the lye.

One does not expect to remove all the lye from all the olives during washing. When 75 to 80 per cent of the olives show no pink or red color with phenolphthalein when this indicator is applied to the cut surface, a satisfactory removal of lye is believed to have taken place.

Fermentation.—For brining and fermentation the olives should be placed in oak barrels. A 44° salinometer (11.66 per cent salt) brine is added at once to Manzanillo, Barouni, or Mission olives. Sevillano and Ascolano olives will shrivel if a concentrated brine is used. Hence Cruess advocates adding a 20° brine to the olives and increasing the concentration by 5° at 2-day intervals until the concentration remains fairly constant at 28 to 30° salinometer. A final concentration of less than 27° salinometer is considered dangerous, for gas tends to form in the olives, likewise spoilage, including softening, increases. The use of a brine more concentrated than 32° produces olives that are too salty in flavor.

The addition of 3 lb. of dextrose, dissolved in the brine, to a 50-gal. barrel increases the acidity of Manzanillo and Mission olives. Sugar should not be added to the other varieties during the fall of the year.

Starters may be added along with the brine, for during the lye treatment a large part of the lactic acid and other bacteria are destroyed. Cruess,[1] experimenting with a number of lactobacilli grown in filtered and sterilized tomato juice, advocates the addition of starters containing pure cultures to green olives. He found *Lactobacillus pentosus* (probably a synonym of *L. plantarum*)[2] to be most desirable, for olives inoculated with this organism possessed a better flavor and higher acidity than uninoculated controls. *L. pentoaceticus* (probably a synonym of *L. brevis*)[2] likewise gave fairly good results. The brine from olives that are

[1] Cruess, W. V., *Fruit Products Jour.*, **17**: 12 (1937).

[2] "Bergey's Manual of Determinative Bacteriology," 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

normal in flavor and odor may be used as a starter. Such brine should contain the fewest possible number of film yeast or mold spores, otherwise its use may cause much more harm than good.

Acidification of the brine with a small amount of vinegar or lactic acid has proved of value in improving the quality of the pickled olives.

Care during Fermentation.—Barrels containing the olives are commonly placed in the sun to ferment, although they may be incubated at a temperature of 70 to 80°F. (21.1 to 26.7°C.). Brine or salt is added several times a week for the first 3 or 4 weeks and thereafter about once a week to maintain a concentration of 28° salinometer and to keep the barrel full. Unless the barrel is kept filled and sealed, film yeast and molds will develop and utilize the acid produced during fermentation. Since the flavor and keeping qualities of the olives depend in large part on the acidity of the brine, the necessity for preventing the growth of the yeast film and molds is evident.

Sugar may be added to the brine if the acidity fails to increase to 0.9 g. or greater per 100 cc.

Packaging.—The olives are packaged in glass jars after the flesh has lost its "chalky-white" color, a characteristic flavor has developed, and the acidity is equivalent to 0.9 g. per 100 cc., or greater. After the jars are filled with olives, water is added to rinse away any sediments. The jars are, after draining, filled then with a 30° salinometer brine, and lactic acid may be added if the original brine contained less than 0.5 g. of acid per 100 cc. Sealing is effected, with or without the use of vacuum.

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CHAPTER XIX

SILAGE

Silage (ensilage) is the fermented product made in silos for cattle food. Although it is most commonly prepared from corn, various other substances, such as sorghum, sunflowers, clover, alfalfa, peas, soybeans, oats, rye, and wheat may be used successfully, provided certain precautions are observed. Forty to sixty pounds of molasses per ton of silage, or green corn, is usually mixed with most of these plants, excepting sorghum and sunflowers.

Procedure.—Corn is harvested and fed into a machine that cuts it into small pieces and blows them to the top of the silo. The inlet at the top of the silo may or may not contain a device for distributing the corn evenly around the silo. The corn is spread uniformly and tramped by men. A fermentation, which is primarily a lactic acid fermentation, ensues.

Harvesting the Corn.—Corn should be harvested[1] when it contains approximately 30 per cent of dry matter. It is usually several weeks after the corn has reached its maximum fresh weight before it contains this amount of dry matter. During the latter part of the growing season, the increase in dry matter occurs principally in the ears.

If the corn contains over 30 per cent of dry matter, it does not usually keep as well in the silo, for, owing to difficulties in cutting it finely, in distributing it in the silo, and in packing it, air spaces are likely to appear. Molds frequently grow in these air spaces, spoiling the product. On the other hand when the dry matter content is much less than 30 per cent, there is usually a loss in the future feeding value of the product.

Packing the Silage.—Silage is packed to exclude the air, which favors mold growth and subsequent spoilage.

If the corn contains more than approximately 35 per cent of dry matter, water should be added to aid in packing it more closely. The incoming corn should be spread evenly around the silo by means of a special distributor as it enters, since ordinarily there is a tendency for the dry, light portions of the corn to accumulate at a point which is farthest away from the corn inlet and since it is difficult to pack such corn without leaving air pockets. It is important, likewise, to guard against uneven settling of the corn in the silo. Accordingly, at least 2 men should tramp the corn.

[1] Nevens, W. B. *Ill. Agr. Expt. Sta., Circ.* 463, November, 1936.

When the corn contains less than 35 per cent of dry matter, packing is not a special problem and it is unnecessary to add water. A higher acidity develops in silage prepared from immature corn.

Fermentation.—During the fermentation that ensues, acids and alcohols are produced, as well as esters. At least 1 per cent of acid is formed, lactic acid being the main acid. Acetic, propionic, butyric, and sometimes formic acids are produced. Besides small amounts of ethyl alcohol, traces of the higher alcohols may be found.

Bacteria, yeasts, and molds may be found in the ensilage. Normal fermentation is brought about by the lactobacilli, especially *Lactobacillus bulgaricus*, while certain other bacteria produce lactic acid in small amounts. The acid formed during fermentation inhibits the development of those types of bacteria which normally bring about putrefactive changes. Members of the colon-aerogenes group of bacteria occasionally produce gas in silage. Yeast-like organisms are found during the early part of the fermentation—they are usually members of the mycoderma group. Molds develop only in the presence of oxygen. Consequently the spoilage of much silage by molds is unnecessary. When it occurs it usually indicates a lack of proper care in packing or covering, or a defective silo.

In some instances, silage has caused serious poisoning when fed to livestock. It has been shown that *Clostridium botulinum* is usually the cause of such forage poisoning.

Lack of sufficient sugar for the proper growth of the lactic acid bacteria, or the development of thermophilic bacteria, in silage sometimes leads to the production of so-called "sweet silage."

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CHAPTER XX

SOME LACTIC ACID BACTERIA AND SOME FERMENTED MILK PRODUCTS

Lactobacillus acidophilus.—*L. acidophilus* has become important in the therapy of various disorders of the gastrointestinal tract, such as constipation, on account of the fact that its successful implantation in the intestines may often relieve the symptoms associated with some of these disorders. When the flora of the intestines is largely putrefactive in nature certain symptoms, such as headaches, may appear. The predominance of *L. acidophilus* in the intestines, along with lactic acid, which it produces, reduces or inhibits the development of the putrefactive types.

It must be emphasized however that, whereas the implantation of *L. acidophilus* has produced very definite relief in certain cases, it is not a cure-all. Successful therapy depends upon careful attention to certain essential details, which will be discussed later.

Description of Organism.—*L. acidophilus* is a member of the important genus, *Lactobacillus*. The bacteria occur as rather slender rods, about 0.6 to 0.9 by 1.5 to 6 μ,[1] singly, in pairs, or in short chains. It is Gram-positive in young cultures but may show Gram-negative forms in older cultures. It is nonsporeforming, nonmotile, microaerophilic, an inhabitant of the intestines of animals, especially herbivores, and has an optimum temperature of 37°C. It produces acid from galactose, glucose, lactose, levulose, maltose, mannose, and sucrose. Some strains ferment raffinose and trehalose, and occasionally dextrin.

There are two main types of the intestinal strain of *L. acidophilus*, although intermediate varieties exist. One produces rough colonies and is referred to as the *X* or *R* type, while the other produces smooth colonies and is recognized as the *Y* or *S* type. Although *R* strains may be readily dissociated to *S* strains, the reverse procedure is very difficult or impossible to achieve.

The strain of *L. acidophilus* that produces rough colonies is of proved therapeutic value and occurs more commonly in the normal human intestines, hence this strain is used commercially in preparing cultures for therapeutic use.

[1] "Bergey's Manual of Determinative Bacteriology," 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

Frequent isolation of the organism from the human intestinal tract favors an active culture that may be implanted readily under favorable conditions.

Conditions Necessary for Implantation.—In order to implant *L. acidophilus*, it is essential to select a strain that will develop in the human intestines. Likewise the organism should be able to multiply rapidly in favorable carbohydrate media and produce a fine coagulum in milk.

Implantation is favored by the ingestion of very large numbers of the organism. When acidophilus milk is used, the dosage recommended is about 1,000 cc. per day. Since the milk must contain at least 100 million viable bacteria per cubic centimeter at the expiration date, it is evident that enormous numbers are consumed.

A second very important factor in successful implantation is the administration of considerable amounts of lactose or dextrin at the time the culture is taken. The use of at least 100 g. of lactose or dextrin daily is advocated, especially if a culture or concentrate of *L. acidophilus* is used rather than milk. Milk, of course, contains lactose as a normal constituent.

L. acidophilus Preparations.—Acidophilus milk is perhaps the most common and preferable preparation. Broth cultures and concentrates of the organism in candy form or combined with yeast have appeared on the market, but are less effective.

Acidophilus Milk.—Acidophilus milk is prepared by inoculating sterilized, fresh whole milk, or partially skimmed milk, with a pure culture of a selected strain of *L. acidophilus*. The milk is sterilized at 120°C. for 15 min. or at lower temperatures for longer periods of time. It is important not to injure the flavor, composition, or appearance of the milk by overtreating it with heat. Pasteurization of milk is not satisfactory, for the types of bacteria that survive pasteurization develop rapidly at the incubation temperature of *L. acidophilus* (35 to 37°C.) and may predominate. The inoculated milk is incubated for 20 to 48 hr. at 35 to 37°C. until a characteristic product is obtained. Usually the fermentation is permitted to proceed until a definite acidity is produced. Some manufacturers[1] consider the fermentation complete when 8 to 10 cc. of N/10 sodium hydroxide solution are required to neutralize a 10-cc. portion of the milk when phenolphthalein is used as the indicator. The lactic acid present in a well-ripened milk is usually 0.65 to 0.75 per cent.

The final product should be slightly sour in flavor and should possess an odor resembling buttermilk. In order to improve the consistency, the product is usually homogenized. It is then bottled and stored at a

[1] "New and Nonofficial Remedies," American Medical n, Chicago, 1937.

temperature of 16 to 20°C.[1] At temperatures of 25°C. and above, the organism continues to produce acid, but eventually the number of viable organisms is reduced. At too low temperatures, the viability of the organism is diminished.

Requirements.[2]—The Council on Pharmacy and Chemistry of the American Medical Association states that broth cultures and concentrates will be acceptable for therapeutic use provided the label states the number of viable organisms in a given quantity at the time of manufacture, provided the label bears an expiration date, and provided the advertising of concentrates and cultures emphasizes the need of the coincidental administrations of such carbohydrates as lactose or dextrin. At the time of manufacture (when the producer has completed the preparation of the product for sale) the preparation must contain at least 200 million viable cells of *L. acidophilus* per cubic centimeter of milk or broth or per gram of concentrate. At the date of expiration, the product must contain not less than 100 million viable cells of *L. acidophilus* per cubic centimeter of milk or broth or per gram of concentrate.

Cultivation.—The organism may be cultivated, for the purpose of study, on casein digest, whey, or tomato-juice agars.[3]

Lactobacillus bulgaricus.—*L. bulgaricus* is an important member of the genus *Lactobacillus*. According to "Bergey's Manual of Determinative Bacteriology,"[4] *L. bulgaricus* (Luerssen and Kühn) Holland is a rod-shaped organism, which often occurs in chains. It is Gram-positive, but older cultures may show unstained portions. It is nonsporeforming, nonmotile, and microaerophilic or anaerobic, usually with an optimum temperature of 45 to 50°C. It is a strong producer of lactic acid and may be isolated from certain fermented milks or dairy products.

L. bulgaricus produces acid from glucose, galactose, and lactose, but not from arabinose, dextrin, inulin, starch, xylose, and certain other carbon-containing compounds.[4] Its action on levulose, maltose, and sucrose is variable or negative depending on the strain used and the conditions of fermentation.

One of the most important uses for *L. bulgaricus* has been in the commercial production of lactic acid. Cultures of this organism have been used to prevent the "wheying off of buttermilk" during the pasteuriza-

[1] Rettger, L. F., M. N. Levy, L. Weinstein, and J. E. Weiss, "Lactobacillus Acidophilus and Its Therapeutic Application," Yale University Press, New Haven, 1935.

Kopeloff, L. M., J. L. Etchells, and N. Kopeloff, *Jour. Bact.*, **28**: 489 (1934).

[2] "New and Nonofficial Remedies," American Medical Association, 1937.

[3] Kopeloff, N., *Psychiat. Quart.*, **9**: 20 (1935).

[4] 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

tion of sour cream[1] and to suppress[2] gas formation in cheeses. It is an important species in some fermented milk products.

Metchnikoff, who advanced the theory that lactic acid bacteria would inhibit the growth of putrefactive types of bacteria in the intestine and thus prevent the formation and absorption of toxic products, believed that *L. bulgaricus* could be established in the intestinal tract of man. His opinion has subsequently been shown to be fallacious, for this organism cannot be successfully implanted. Consequently it is considered to have no therapeutic value. It is to be noted, however, that at the time of Metchnikoff's interest in this subject, no distinction between *L. bulgaricus* and *L. acidophilus* had been made. The organism described by him as *L. bulgaricus* may have been a mixed culture of the two species.

Some Other Fermented Milks. *Buttermilks.*—Buttermilks may be classified as natural or artificial buttermilks. Natural buttermilk may be defined as the product that remains when fat is removed from sour milk or cream in the process of churning. It may be obtained from naturally soured cream or cream that has been pasteurized and ripened with a special butter starter. Natural buttermilk, prepared from naturally soured cream, will contain a varied flora. Consequently the flavor of such products will depend largely upon the care used in their production. A typical buttermilk may contain approximately 0.2 per cent fat, 4.4 per cent lactose, 3.5 per cent protein, 0.7 per cent ash, 0.6 per cent lactic acid and 90 per cent water.

Artificial buttermilk, known also as cultured buttermilk, is "the product obtained by souring pasteurized skimmed or partially skimmed milk by means of a suitable culture of lactic bacteria. It contains not less than 8.5 per cent of milk solids not fat."[3] *Streptococcus lactis*, *L. bulgaricus*, and *Strept. citrovorus* or *Strept. paracitrovorus* or a combination of the first two of these bacteria may be used to inoculate the pasteurized milk. The milk is incubated at an optimum temperature for the fermentation (about 37°C.) until an acidity of approximately 0.75 to 0.85 per cent is obtained. The product is then agitated to yield a smooth and creamy buttermilk.

Since some persons object to the rather thick consistency of artificial buttermilk, skim milk, whole milk, or sweet cream may be added after the fermentation to improve the consistency. In some instances, salt, fruit juices, or extracts may be added to modify buttermilks, which are then bottled and stored at relatively low temperatures to prevent increases in acidity and undesirable changes in flavor or consistency.

[1] *Ill. Agr. Expt. Sta., Circ.* 166, 1913.

[2] *U.S. Dept. Agr., Bull.* 148, 1915.

[3] *U.S. Dept. of Agriculture*, F.D.A., Service and Regulatory Announcements, Food and Drug, 2, *Rev.* 5, November, 1936.

Kefir is a fermented milk product that originated in the Caucasus Mountains. It is extensively used as a food by the natives of that region. It is prepared by inoculating the milk of mares, goats, ewes, or cows with small convoluted masses of "seed," known as kefir grains. These grains resemble to some extent miniature cauliflowers and may be dried and preserved. Kefir grains usually contain *Lactobacillus casei*, streptococci, and a lactose-fermenting yeast, *Saccharomyces kefir*, besides other microorganisms. Since the fermentation is usually carried out in goatskin bags or closed glass bottles, the product becomes effervescent, owing to the evolution of gas. Lactic acid and alcohol are two important fermentation products.

Kefir has also been known as *kefr*, *kephir*, and *kephyr*.

A product closely related to the original kefir is made by adding sucrose (about 4 per cent) and a culture of yeast to buttermilk in a covered glass bottle. It is incubated at 70°F. (21.1°C.) until a desirable end point is reached and is then cooled to stop the growth of the organisms.

Yoghurt is a fermented milk product used by Bulgarian tribes. It is primarily a soured milk but occasionally may contain some alcohol. *Lactobacillus bulgaricus* is the dominating organism found in yoghurt, although it is the product of a mixed flora of microorganisms.

Metchnikoff, who was amazed at the longevity of the tribes that used yoghurt regularly, studied this fermentation and publicized an account of it in his book, "The Prolongation of Life."

Yoghurt is likewise known as *yogurt, yoghourt, yahourth, yohourt,* or *yahourd.*

Kumiss is a fermented milk product that originated in a Russian tribe, known as Kumanes, living near the Kuma River. It is usually prepared from mare's or cow's milk and contains lactic acid and alcohol. The fermentation, brought about by a mixed flora, in which, according to Rogers,[1] lactic streptococci, lactobacilli of the *L. casei* type, and a lactose-fermenting yeast are the predominating organisms, is carried out in skin or leathern bags or in open vessels. If the fermentation proceeds in a closed container an effervescent product is obtained, owing to the retention of the gas evolved.

Kumiss is sometimes known as *kumyss, kumys,* or *koumiss.*

Leben is a fermented product made usually from cow's, goat's, or buffalo's milk. Bacteria and yeasts ferment the milk to produce acid and alcohol. Although this product has been native to Egypt for hundreds of years, where it is known as *leben* or *leben raib,* it is used in Syria, Sardinia, Algeria, and in other regions.

[1] Rogers, L. A. (associates of), "Fundamentals of Dairy Science," 2d ed., Reinhold Publishing Corporation, New York, 1935.

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CHAPTER XXI

CHEESE

Cheese was prepared in Asia and in Europe several hundred years, at least, before the birth of Christ. A study of the origin of some of the many different types of cheeses lies outside the scope of this text, but the interested reader will derive considerable enjoyment and add to his knowledge by perusing the history of cheese making.

Definition.—Cheese, according to the definition and standards of the Food and Drug Administration of the U.S. Department of Agriculture,[1] is:

. . . the product made from the separated curd obtained by coagulating the casein of milk, skimmed milk, or milk enriched with cream. The coagulation is accomplished by means of rennet or other suitable enzyme, lactic fermentation, or by a combination of the two. The curd may be modified by heat, pressure, ripening ferments, special molds, or suitable seasoning.

The name "cheese" unqualified means Cheddar cheese (American cheese, American cheddar cheese).

The Milk.—Milk from ewes (sheep), goats, cows, mares, and other animals has been used for the making of cheeses. Each of these milks contains fats, protein, milk sugar (lactose), mineral salts, and water. Fat is present as an emulsion, but the sugar, minerals, and some of the proteins are soluble in the water of milk. Casein (the principal protein) is combined with calcium and exists in a colloidal condition.

Formation of Curd.—If acid is produced in milk as a result of the fermentation of lactose by bacteria, the casein is freed from calcium and no longer appears as fine particles distributed throughout the medium, but instead goes out of solution and eventually accumulates in large lumps of curd or as a continuous mass.

Rennet, an enzyme, likewise has the ability to alter the colloidal condition of casein, causing coagulation or curdling. This enzyme, sometimes known as "rennin," is obtained commercially by extracting the fourth stomach of the calf where it is principally found. Commercial pepsin sometimes serves as a successful substitute for rennet.

Whey.—Whey is the watery portion of milk separated from the curd during cheese making. The principal constituents of whey are lactose,

[1] Service and Regulatory Announcements, Food and Drug, No. 2, Rev. 5, Nov. 5, 1936.

soluble ash, and lactalbumin. A small amount of fat is a normal component of whey also.

Treatment of Curd.—The curd formed in milk through the action of rennet, lactic acid, or a combination of the two, may be treated in a variety of manners. After it has settled into a fairly compact jelly-like mass, it is usually cut into small pieces by special knives to facilitate drainage or removal of occluded whey. It may be drained with or without eventual pressure, depending on the type of cheese being manufactured. The curds of the soft or semisoft cheeses are pressed just sufficiently to remove the excess of whey, while those of the hard cheeses may be heated and pressed in a form with weights until the curd forms a firm mat. The curd is treated with varying quantities of salt by various methods. Finally the treated curd is molded into the desired shape and is ready for ripening or curing.

Ripening.—During this process, the flavor, composition, and physical properties of the cheese undergo change due to the action of characteristic molds or bacteria and their enzymes. The nature of the changes depends on the method used in treating the curd, the method and quantity of salting, the microorganisms present or added, and the control of temperature and relative humidity in the curing rooms, chambers, or caves.

Classification.—Cheeses may be classified in several manners: on the basis of hardness, as hard, semihard, and soft cheeses; on the basis of the

TABLE 63.—A CLASSIFICATION OF CHEESES

Name of Cheese	Place of Origin	Name of Cheese	Place of Origin
Hard Rennet Cheeses		Semihard Rennet Cheeses	
Cheddar	England	Limburger	Belgium
Cheshire (Cheddar type)	Chester, England	Stilton (mold)	Stilton, England
Caciocavallo	Southern Italy	Brie (mold)	France
Gorgonzola (mold)	Italy (near Gorgonzola)	Blue (mold)	France, Denmark, etc.
Parmesan	Italy	Camembert (mold)	France
Pecorino	Italy	Roquefort (mold)	France
Provolono	Italy	Münster	Near Münster, Germany
Reggiano	Italy	Brick	United States
Romano	Italy	Soft Rennet Cheeses	
Edam	Edam, Holland	Port du Salut	Canada
Gjedost	Norway	Neufchâtel	France
Emmenthaler	Switzerland	Soft Lactic Cheeses	
Gruyère (Swiss)	Gruyère, Switzerland	Cottage	
Pineapple	United States	Cream	United States

principal ripening agents, as mold or bacterial cheeses; on the basis of the kind of milk from which manufactured, as cow, ewe, goat, or other milk; on the basis of the country of origin, as Italian, French, American, and the like; on the basis of the method of coagulation, as rennet or acid curd cheese; on the basis of added substances, such as sage; or on the basis of the fat content of the milk, as full cream, whole milk, or skim-milk cheeses.

In Table 63 cheeses are classified as hard, semihard, and soft rennet cheeses, and as lactic cheeses. Sometimes Roquefort and Stilton cheeses are classified as hard cheeses and Brick, Brie, Camembert, Limburger, and Münster as soft cheeses, rather than as semihard cheeses. Blue cheese, Brie, Camembert, Gorgonzola, Roquefort, and Stilton are characterized as mold cheeses.

Analyses of Some Varieties of Cheese.—Table 64 contains the average analyses of some varieties of cheese.

Cheddar Cheese.—Cheddar cheese, a hard cheese prepared from whole milk by the action of rennet, originated in Cheddar, England. It is the leading cheese manufactured in the United States and is widely made by the English.

The milk used for the manufacture of cheddar cheese is analyzed, standardized, weighed, and heated to about 86°F. (30°C.), and a suitable starter of lactic acid bacteria is added. Vegetable coloring matter may be incorporated also at this time if it is to be used. The milk is thoroughly mixed, and when the acidity is correct (0.17 to 0.2 per cent) rennet (approximately 3 oz. for each 1,000 lb. of milk), diluted with water, is added. It requires only 20 to 40 min. to coagulate the casein. When coagulation is satisfactory, demonstrated by a clean breaking of the curd, leaving a clear whey, the curd is cut by special knives into cubes about ¼ in. square to permit the whey to escape more readily. The curd is slowly heated to about 100°F. (37.8°C.). Acidity and body are developed in the curd as it is cooked with gentle agitation to keep it from settling. The whey is drained off when the curd has assumed the desired texture.

The curd is now cut into large pieces, which are turned frequently and placed one on top of the other to force out the whey. This treatment is known as cheddaring.

Cheddared curd is then cut up and milled into small pieces by special machines. Salt, at the rate of approximately 1.5 lb. to each 1,000 lb. of curd, is added and carefully mixed with the curd. The product is cooled to 80°F. (26.7°C.), packed in hoops containing cheesecloth linings, and pressed for several hours to the desired shape.

During the ripening process, originally carried out in the Cheddar caves and which may require a few weeks or several months, the bacteria

TABLE 64.—AVERAGE ANALYSES OF SOME VARIETIES OF CHEESE[1]

Variety	Authority	Water, per cent	Fat, per cent	Proteides, amides, etc., per cent	Milk sugar, lactic acid, etc., per cent
Brick.	Bureau of Chemistry	42.47	30.66	21.05	
Brie:					
Imported.	Bureau of Chemistry	52.53	22.44	20.94	
American	Bureau of Chemistry	52.10	24.72	19.60	
Caciocavallo	Bureau of Chemistry	34.95	21.98	34.33	
Camembert, imported	Bureau of Chemistry	47.88	26.32	22.21	
Cheddar skim	Bureau of Chemistry	57.04	4.88	32.09	
Cream-French, Demi-sel.	Bureau of Chemistry	42.74	39.88	14.49	
Edam (American)	Bureau of Chemistry	38.07	22.65	30.89	
Emmenthaler	Lindt	33.00	30.50	30.44	
Gorgonzola	Musso	37.30	34.67	25.16	1.62
Gouda	Cribb	54.79	9.02	25.94	....
Gruyère	Balland	29.99	28.19	33.03	4.82
Limburger:					
American	Arnold	35.64	29.82	28.53	
Imported	Bureau of Chemistry	54.79	19.61	21.27	
Münster (American)	Bureau of Chemistry	40.60	31.00	22.20	
Neufchâtel	Bureau of Chemistry	52.05	23.51	19.33	
American	Bureau of Chemistry	59.22	18.17	21.30	
Parmesan:					
Formaggio	Bureau of Chemistry	16.95	22.71	49.39	
Reggiano	Bureau of Chemistry	29.63	27.29	34.84	
Pecorino	Sartori	29.80	30.51	33.51	
Romano	Bureau of Chemistry	29.56	27.69	31.20	
Pineapple	Johnson	24.07	38.12	29.35	2.49
Port du Salut	Bureau of Chemistry	50.10	25.17	21.18	
Roquefort	Currie	38.69	32.31	21.39	
Stilton	Bureau of Chemistry	33.57	31.19	28.96	
Swiss:					
American	Bureau of Chemistry	34.28	32.60	27.55	
Imported	Bureau of Chemistry	33.91	30.61	29.22	

[1] Data tabulated from *U.S. Dept. Agr., Bull.* 608, revised February, 1932.

and enzymes present bring about characteristic changes in the cheese. Lactic acid bacteria, such as *Streptococcus lactis* and *Lactobacillus casei*, are especially important in the ripening or curing of the cheddar cheese. The bacteria increase enormously, often reaching several hundred millions in numbers per gram during the first few weeks, and then gradually die off until only a few million per gram remain at the end of 8 months.

It is necessary to prevent molds from growing on this cheese, for they cause a rapid deterioration of the product. This is done by use of salt on the surface.

A yield of approximately 1 lb. of cheese is obtained from 10 lb. of milk by this process.

Roquefort Cheese.—Roquefort cheese is a semihard, friable cheese characterized by a mottled or marbled appearance of the interior, due to the presence of *Penicillium roqueforti*, a blue-green mold. This cheese has been known for almost a thousand years, having originated in the south of France. It is made principally from the milk of ewes, bred particularly for their high milk-producing abilities. The sheep graze in the plateau region of the Cevennes. Their milk is made into cheese in small factories distributed over this relatively small section of Southern France. The cheese is transported to the vicinity of Roquefort for ripening.

In making Roquefort cheese, the curd is drained in hoops but not pressed. It is inoculated at this time with a dried bread product rich in mold spores, the inoculum being prepared by growing *P. roqueforti* in the interior of bread in a moist cool place until the bread becomes a moldy mass containing a vast number of spores. The bread is dried carefully and ground to a powder, the latter being used as the inoculum.

During ripening, the temperature and relative humidity must be carefully controlled in order to obtain a fine cheese. The temperature of ripening must not be much higher than 48°F. (8.9°C.), and the relative humidity should be high. In France, the ripening is carried out in natural caves and in artificial caves hewn from the limestone. Water trickling down through crevices cools the air and at the same time nearly saturates it. The flow of air through the caves is regulated in order to obtain the desired temperature and humidity. In the United States, Roquefort-type cheese is made in curing rooms having regulated relative humidity and temperature; in caves; and, in one instance at least, in a mine shaft.

Holes are punched into the curd to facilitate the development of the mold throughout the cheese. Although aerobic, *P. roqueforti* will grow with a minimum supply of air. After the mold has developed to the desired extent, the cheese is wrapped in tinfoil and stored at approxi-

mately 40°F. (4.44°C.), at which temperature the enzymes of the mold are active, but the mold growth is inhibited.

The pungent taste of Roquefort is due in part to the action of lipase, a fat-splitting enzyme, which liberates caproic, caprylic, and capric acids from the fat.

Roquefort Cheese from Cow's Milk.—Roquefort cheese has been manufactured on a commercial basis from cow's milk since 1918. Roquefort cheese made from cow's milk is, usually, of a more yellow color than the cheese made from sheep's milk.

Other Cheeses.—Brief descriptions and analyses of many varieties of cheese will be found in *Bulletin* 608 of the U.S. Department of Agriculture.

For a full discussion of the various cheese-making processes, the reader is referred to the publications cited in the bibliography at the end of the chapter.

The U.S. Department of Agriculture has adopted the following definitions and standards for cheese.[1]

WHOLE MILK CHEESE

Cheddar Cheese, American Cheese, American Cheddar Cheese. The cheese made by the Cheddar process from heated and pressed curd obtained by the action of rennet on whole milk. It contains not more than 39 percent of water, and, in the water-free substance, not less than 50 percent of milk fat.

Pineapple Cheese. The cheese made by the pineapple Cheddar cheese process from pressed curd obtained by the action of rennet on whole milk. The curd is formed into a shape resembling a pineapple, with characteristic surface corrugations, and during the ripening period the cheese is thoroughly coated and rubbed with a suitable oil, with or without shellac. The finished cheese contains, in the water-free substance, not less than 50 percent of milk fat.

Brick Cheese. The quick-ripened cheese made by the brick-cheese process from pressed curd obtained by the action of rennet on whole milk. It contains, in the water-free substance, not less than 50 percent of milk fat.

Stilton Cheese. The cheese made by the Stilton process from unpressed curd obtained by the action of rennet on whole milk, with or without added cream. During the ripening process a special blue-green mold develops, and the cheese thus acquires a marbled or mottled appearance in section.

Gouda Cheese. The cheese made by the Gouda process from heated and pressed curd obtained by the action of rennet on whole milk. The rind is colored with saffron. The finished cheese contains, in the water-free substance, not less than 45 percent of milk fat.

Neufchâtel Cheese. The cheese made by the Neufchâtel process from unheated curd obtained by the combined action of lactic fermentation and rennet on whole milk. The curd, drained by gravity and light pressure, is kneaded or

[1] F.D.A., Service and Regulatory Announcements, Food and Drug No. 2, Rev. 5, November, 1936.

worked into a butter-like consistence and pressed into forms for immediate consumption or for ripening. The finished cheese contains, in the water-free substance, not less than 50 percent of milk fat.

Cream Cheese. The unripened cheese made by the Neufchâtel process from whole milk enriched with cream. It contains, in the water-free substance, not less than 65 percent of milk fat.

Roquefort Cheese. The cheese made by the Roquefort process from unheated, unpressed curd obtained by the action of rennet on the whole milk of sheep, with or without the addition of a small proportion of the milk of goats. The curd is inoculated with a special mold (*Penicillium roqueforti*) and ripens with the growth of the mold. The fully ripened cheese is friable and has a mottled or marbled appearance in section.

Gorgonzola Cheese. The cheese made by the Gorgonzola process from curd obtained by the action of rennet on whole milk. The cheese ripens in a cool, moist atmosphere with the development of a blue-green mold and thus acquires a mottled or marbled appearance in section.

WHOLE MILK OR SKIM MILK CHEESE

Edam Cheese. The cheese made by the Edam process from heated and pressed curd obtained by the action of rennet on whole milk or on partly skimmed milk. It is commonly made in spherical form and coated with a suitable oil and a harmless red coloring matter.

Swiss Cheese. The cheese made by the Emmenthaler process from heated and pressed curd obtained by the action of rennet on whole milk or on partly skimmed milk. It is ripened by special gas-producing bacteria, causing characteristic "eyes" or holes. The finished cheese contains, in the water-free substance, not less than 45 percent of milk fat.

Camembert Cheese. The cheese made by the Camembert process from unheated, unpressed curd obtained by the action of rennet on whole milk or on slightly skimmed milk. It is ripened by the growth of a special mold (*Penicillium camemberti*) on the outer surface. The finished cheese contains, in the water-free substance, not less than 45 percent of milk fat.

Brie Cheese. The cheese made by the Brie process from unheated, unpressed curd obtained by the action of rennet on whole milk, on milk with added cream, or on slightly skimmed milk. It is ripened by the growth of a special mold on the outer surface.

Parmesan Cheese. The cheese made by the Parmesan process from heated and hard-pressed curd obtained by the action of rennet on partly skimmed milk. The cheese, during the long ripening process, is coated with a suitable oil.

Cottage Cheese. The unripened cheese made from heated or unheated, separated curd obtained by the action of lactic fermentation or rennet, or a combination of the two, on skimmed milk, with or without the addition of buttermilk. The drained curd may be enriched with cream, and salted or otherwise seasoned.

PASTEURIZED CHEESE

Pasteurized Cheese, Pasteurized-blended Cheese. The pasteurized product made by comminuting and mixing, with the aid of heat and water, one or more

lots of cheese into a homogeneous, plastic mass. The unqualified name "pasteurized cheese," "pasteurized-blended cheese," is understood to mean pasteurized Cheddar cheese, pasteurized-blended Cheddar cheese, and applies to a product which conforms to the standard for Cheddar cheese. Pasteurized cheese, pasteurized-blended cheese, bearing a varietal name is made from cheese of the variety indicated by the name and conforms to the limits for fat and moisture for cheese of that variety.

PROCESS CHEESE

Process Cheese. The modified cheese made by comminuting and mixing one or more lots of cheese into a homogeneous, plastic mass, with the aid of heat, with or without the addition of water, and with the incorporation of not more than 3 percent of a suitable emulsifying agent. The name "process cheese" unqualified is understood to mean process Cheddar cheese, and applies to a product which contains not more than 40 percent of water and, in the water-free substance, not less than 50 percent of milk fat. Process cheese qualified by a varietal name is made from cheese of the variety indicated by the name, and conforms to the limits for fat and moisture for cheese of that variety.

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CHAPTER XXII

THE PROPIONIC ACID FERMENTATION

Historical.—In 1841 Nöllner[1] isolated a "Pseudo-Essigsäure" (pseudo-acetic acid) from the decomposition products of tartaric acid. This substance is believed to have been largely propionic acid (CH_3CH_2COOH).

Fitz, in a series of articles on fermentation,[2] discussed the morphology and biochemical activities of the bacteria producing propionic acid from calcium malate and from calcium lactate.

Orla-Jensen[3] (1898) carried out research concerning the formation of eyes in Emmenthaler cheese. Later von Freudenreich and Orla-Jensen[4] reported the first description of the isolation of microorganisms causing the propionic acid fermentation. They studied the propionic acid bacteria rather extensively, especially in relation to cheese.

As the result of investigations carried out to discover the reasons why Emmenthaler cheese manufactured in the United States was lacking in characteristic flavor and why the eye formation was deficient or abnormal, Sherman and Shaw[5] supplied much important information in respect to the propionic acid bacteria; for example, they demonstrated the relationship between the propionic acid bacteria and the production of a high-grade Emmenthaler cheese. Sherman (1924) described the use of pure cultures of propionic acid bacteria, *Bacterium acidi-propionici d*, to ensure the production of Emmenthaler cheese with characteristic flavor and with normal eye development. This organism did not suppress the growth of undesirable bacteria, however. Cultures of *Lactobacillus bulgaricus* have long been used in cheese making to inhibit the development of the wrong types of bacteria.

Whittier and Sherman[6] studied the factors affecting the propionic acid fermentation, and, with Albus, the rates of fermentation of lactose, galactose, glucose, sucrose, and maltose.[7]

[1] Nöllner, C., *Ann.*, **38**: 299 (1841).

[2] Fitz, A., *Ber.*, **9**: 1348 (1876); **10**: 276 (1877); **11**: 42, 1890 (1878); **12**: 474 (1879); **13**: 1309 (1880); **14**: 1084 (1881); **15**: 867 (1882); **16**: 844 (1883); **17**: 1188 (1884).

[3] Orla-Jensen, S., *Centr. Bakt. Parasitenk.*, Abt. II, **4**: 217, 265, 325 (1898).

[4] Freudenreich, E. von, und Orla-Jensen, S., *Centr. Bakt. Parasitenk.*, Abt. II, **17**: 529 (1906).

[5] Sherman, J. M. and R. H. Shaw, *Jour. Biol. Chem.*, **56**: 695 (1923).

[6] Whittier, E. O., and J. M. Sherman, *Ind. Eng. Chem.*, **15**: 729 (1923).

[7] Whittier, E. O., J. M. Sherman, and W. R. Albus, *Ind. Eng. Chem.*, **76**: 122 (1924).

Virtanen[1] has studied the mechanism of the propionic acid fermentation.

Van Niel's[2] dissertation on the propionic acid bacteria was published in 1928. This comprehensive monograph will prove to be of much value to anyone interested in the propionic acid bacteria.

Werkman, Wood, Stone, and their associates have carried out considerable research on the propionic acid fermentation, particularly in respect to the mechanism of the fermentation. Some of their work will be discussed later.

Tatum, Peterson, and their coworkers[3] have studied the effect of growth factors on propionic acid bacteria.

Factors Affecting the Fermentation. *The Organism.*—Propionic acid bacteria, in general, may be characterized as Gram-positive, catalase positive, nonsporeforming, nonmotile, facultative aerobes.

Van Niel[4] lists eight main species (with their synonyms) on the basis of morphological, cultural, and biochemical differences:

Propionibacterium freudenreichii (*Bacterium acidi propionici a* von Freudenreich and Orla-Jensen, etc.)
P. jensenii (*Bact. acidi propionici b* von Freudenreich and Orla-Jensen)
P. peterssonii (*Bact. acidi propionici c* Troili Petersson)
P. shermanii (*Bact. acidi propionici d* Sherman)
P. pentosaceum (*Bacillus acidi propionici* von Freudenreich and Orla-Jensen)
P. rubrum (*Bact. acidi propionici* var. *rubrum.* Thoeni et Allemann)
P. thoenii (*Bact. acidi propionici* var. *rubrum.* Thoeni et Allemann)
P. technicum

The following additions to this list have been suggested:

P. raffinosaceum Werkman and Kendall[5]
P. arabinosum Hitchner[6]
P. zeae Hitchner[6]

P. technicum has the ability to ferment starch, dextrin, and glycogen—other previously described propionic acid bacteria did not possess this ability. *P. thoenii* produces propionic acid and acetic acid from a glucose medium containing yeast extract in a molecular ratio of approximately 5:1, according to Van Niel.

[1] Virtanen, A. I., *Soc. Sci. Fennica, Commentationes Phys. Math.*, **1** (No. 36): 1 (1923); **2** (No. 20): 1 (1925).

[2] Van Niel, C. B., "The Propionic Acid Bacteria," Technische Hoogeschool, Delft, September, 1928.

[3] Tatum, E. L., W. H. Peterson, and E. B. Fred, *Jour. Bact.*, **32**: 157 (1936); Tatum, E. L., H. G. Wood, and W. H. Peterson, *Jour. Bact.*, **32**: 167 (1936); Snell, E. E., F. M. Strong, and W. H. Peterson, *Jour. Bact.*, **38**: 293 (1939).

[4] Van Niel, *loc. cit.*

[5] Werkman, C. H., and S. E. Kendall, *Iowa State Coll. Jour. Sci.*, **6**: 17 (1931).

[6] Hitchner, E. R., *Jour. Bact.*, **23**: 40 (1932); **28**: 473 (1934).

Propionic acid bacteria may be isolated from a number of sources: milk, cheeses, and other dairy products; silage; soil; the excreta of cattle; and other sources.

The Carbon Source.—A large number of raw materials have been utilized as sources of carbon by different species of *Propionibacterium.* Some materials fermented include: lactose, sucrose, maltose, glucose, raffinose, arabinose, xylose, glycogen, dextrin, and starch; lactic, tartaric, and quinic acids; glycerol and mannitol; and proteins, protein derivatives, and fats. Lactose and low-priced carbohydrates would undoubtedly be used in the industrial production of propionic acid.

The Nitrogen Source.—Propionic acid bacteria may utilize several nitrogen-containing compounds. One of the most satisfactory nitrogen sources is yeast extract, at a concentration of approximately 0.4 per cent. Peptones, whey, and corn meal may be utilized, especially in the association of other bacteria, such as *Proteus mirabilis,*[1] *Streptococcus lactis,*[2] or *Lactobacillus casei.*[2]

Sherman suggests that the increased production of propionic acid resultant from the association of bacteria may be due to the fact that lactic acid is more readily utilized than lactose by the propionic acid bacteria. Van Niel suggests that the stimulating effect may be due to an alteration in the nitrogenous compounds of the medium.

Tatum and his associates[3] state that the propionic acid bacteria may be able to utilize ammonia as the only source of nitrogen when suitable stimulatory substances are present. They suggest that yeast extract and other complex nitrogen sources may be effective because they contain nonnitrogenous growth factors in addition to available nitrogen.

The nitrogen source is of much importance for it influences the rate and the completeness of fermentation, also the ratio of propionic acid to acetic acid.

Growth Factors.—It has been stated repeatedly in some of the earlier literature that a complex source of nitrogen was essential for the growth of propionic acid bacteria. Recent research has indicated that growth factors rather than complex nitrogen sources are most important.

According to Wood and his associates,[4] amino acids are beneficial but not essential for the growth of propionic acid bacteria.

Wood and his coworkers[5] obtained an ether-soluble factor from yeast extract, which was indispensable for the growth of all the cultures of propionic acid bacteria tested on a synthetic medium that contained

[1] Van Niel, *loc. cit.*

[2] Sherman, J. M. and R. H. Shaw, *Jour. Gen. Physiol.,* **3**: 657 (1921).

[3] Tatum, Wood, and Peterson, *loc. cit.*

[4] Wood, H. G., A. A. Andersen, and C. H. Werkman, *Jour. Bact.,* **36**: 201 (1938).

[5] Wood, H. G., E. L. Tatum, and W. H. Peterson, *Jour. Bact.,* **33**: 227 (1937).

ammonium sulphate as the nitrogen source. This factor has been found in potato extract, corn extract, corn steep, and liver extract, in addition to yeast extract. It is a nonvolatile acid, soluble in chloroform, benzene, ether, and xylene but almost insoluble in petroleum ether. It may be adsorbed on Norit and eluted with acid-alcohol. The factor is not replaced by a mixture containing vitamin B_1, nicotinic acid, pimelic acid, uracil, beta-alanine, and pantothenic acid.

Vitamin B_1 (thiamin) stimulated the growth of propionic acid bacteria, especially in the presence of amino acids. But not all propionic acid bacteria require Vitamin B_1 for vigorous growth, according to Tatum and his associates.[1]

Riboflavin,[2] in a concentration of 0.05 gamma per cubic centimeter, stimulated the growth of propionic acid bacteria in a medium containing ammonium sulphate.

For a further discussion of this subject the reader is urged to consult the various papers on growth factors that are cited at the end of this chapter.

The pH.—Most favorable results are obtained when the pH is adjusted to 6.8 to 7.2, a pH of 7.0 being usually preferred.

The Temperature.—The optimum temperature for fermentation is approximately 30°C.

Duration of Fermentation.—The fermentation normally requires 7 to 12 days, but, by adapting the fermentation to a semicontinuous basis, Van Niel demonstrated that the fermentation time may be considerably reduced.

Composition of Some Culture Media.—Van Niel[3] used a culture medium containing yeast extract and 2 per cent sodium lactate, adjusted to a pH of 7 in some of his researches. Another medium used by him contained yeast extract, 2 per cent glucose, and 2 per cent calcium carbonate.

Tatum, Peterson, and Fred[4] have cultured the organisms on a medium containing 1 per cent malt sprouts, 1 per cent glucose, and calcium carbonate.

A medium consisting of 5 g. of lactose, 5 g. of calcium carbonate, and 1 g. of dried yeast in 100 cc. of water, with the pH adjusted to approximately 7, was used by Whittier and Sherman[5] under certain conditions.

[1] Tatum, E. L., H. G. Wood, and W. H. Peterson, *Biochem. Jour.*, **30**: 1898 (1936).

[2] Wood, H. G., A. A. Andersen, and C. H. Werkman, *Proc. Soc. Exptl. Biol. Med.*, **36**: 217 (1937); Lava, V. G., R. Ross, and K. C. Blanchard, *Philippine Jour. Sci.*, **59**: 493 (1936).

[3] Van Niel, *loc. cit.*

[4] Tatum, Peterson, and Fred, *op. cit.*

[5] Whittier and Sherman, *loc. cit.*

A basal medium containing 1 per cent glucose, 0.6 per cent sodium lactate, 0.3 per cent ammonium sulphate, and Speakman's inorganic salts in half concentration[1] (0.25 g. K_2HPO_4, 0.25 g. KH_2PO_4, 0.1 g. $MgSO_4 \cdot 7H_2O$, 0.005 g. NaCl, 0.005 g. $FeSO_4 \cdot 7H_2O$, 0.005 g. $MnSO_4 \cdot 4H_2O$, and 1,000 cc. water)[2] was used by Wood and his coworkers in some of their research.

Products of the Fermentation.—The main end products of the propionic acid fermentation are propionic acid, acetic acid, and carbon dioxide. Small amounts of succinic acid are frequently produced, while acetylmethylcarbinol is occasionally formed by certain species.

Ratio of Propionic Acid to Acetic Acid.—The ratio of propionic to acetic acid varies according to the species, the nitrogen source, and other factors. In a medium containing glucose and yeast extract, the acids were produced in a ratio of approximately 5:1 by *Propionibacterium thoenii*. Under similar conditions the acids were formed in a ratio of about 3:1 by *P. rubrum*.[3] When using *P. shermanii* (*Bacterium acidi propionici d*), Whittier and Sherman observed a fairly constant ratio of 2 molecules of propionic acid to 1 molecule of acetic acid.[4]

Yields.—Usually more than 75 per cent of the fermented sugar may be accounted for as propionic and acetic acids, while less than 20 per cent is used for carbon dioxide production.

In one experiment, in which the medium contained yeast extract, 2 per cent glucose, and 2 per cent calcium carbonate, Van Niel obtained the following results: 13.24 g. glucose fermented; and 8.62 g. propionic acid, 1.85 g. acetic acid, 1.363 g. carbon dioxide, and 0.45 g. succinic acid produced.

Whittier[4] and Sherman have determined the conditions whereby yields of approximately 2.4 lb. of propionic acid and 1 lb. of acetic acid may be obtained from 5 lb. of lactose after a fermentation period of 12 days at 30°C., using a mixed culture of *P. shermanii* and *Lactobacillus casei* as the inoculum. Yields of 85 per cent or greater usually required a fermentation period of 2 weeks or longer.

Uses.—The propionic acid bacteria determine the taste and flavor as well as the characteristic eye development in Emmenthaler cheese.

Propionic acid is used in the manufacture of perfumes. Ethyl propionate is a solvent for pyroxylin. The mixture of propionic and acetic acids may be distilled to produce a mixture of acetone, methylethyl ketone, and diethyl ketone,[4] substances which are solvents.

Large quantities of propionic acid could be manufactured industrially by fermentation, provided that a demand arose for the acid.

[1] Wood, H. G., A. A. Andersen, and C. H. Werkman, *Jour. Bact.*, **36**: 201 (1938).
[2] Wood, Tatum, and Peterson, *loc. cit.*
[3] Van Niel, *loc. cit.*
[4] Whittier and Sherman, *loc. cit.*

The Mechanism of the Propionic Acid Fermentation.—Fitz[1] proposed that lactic acid was converted by propionic acid bacteria in accordance with the following equation:

$$\begin{array}{ccccccc} CH_3 & & CH_3 & & & & \\ OH & = 2 & CH_2 & + & CH_3 & + & CO_2 + H_2O \\ | & & | & & | & & \\ COOH & & COOH & & COOH & & \\ \text{Lactic acid} & & \text{Propionic acid} & & \text{Acetic acid} & & \end{array}$$

Virtanen,[2] Virtanen and Karström,[3] and Van Niel[4] were of the opinion that two 3-carbon molecules were formed from glucose after phosphorylation, one of these being oxidized to acetic acid and carbon dioxide, while two other molecules were being reduced to propionic acid.

$$\text{Glucose} \rightarrow \text{Hexosephosphate} \rightarrow \begin{array}{c}\text{Two 3-carbon—} \\ \text{compounds}\end{array} \begin{array}{l} \xrightarrow{\text{Oxidation}} \begin{array}{l}\text{Acetic acid +} \\ \text{Carbon dioxide}\end{array} \\ \\ \xrightarrow[\text{Reduction}]{} \text{Propionic acid} \end{array}$$

Van Niel proposed that pyruvic acid acted as an intermediate.

Virtanen[2] assumed that succinic acid arose in the following manner from glucose:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Succinic acid}}{\begin{array}{c} CH_2{\cdot}COOH \\ | \\ CH_2{\cdot}COOH \end{array}} + \underset{\text{Acetaldehyde}}{\begin{array}{c} CH_3 \\ | \\ CHO \end{array}} + H_2O$$

Wood, Stone, and Werkman[5] have proposed the following scheme for the dissimilation of glucose by propionic acid bacteria:

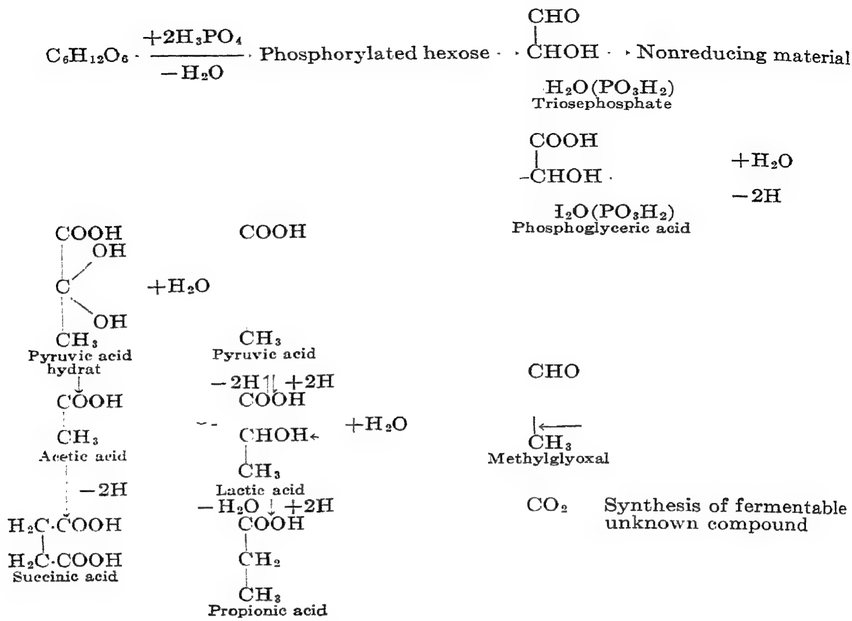
[1] Fitz, A., *Ber.*, **11:** 1896 (1878).

[2] Virtanen, *loc. cit.*

[3] Virtanen, A. I., and H. Karström, *Acta Chem. Fennica, Ser. B*, **7:** 17 (1931).

[4] Van Niel, *Op. cit.*

[5] Wood, H. G., R. W. Stone, and C. H. Werkman, *Biochem. Jour.*, **31:** 349 (1937).



Some Possible Evidence for Foregoing Schemes.—Several compounds that may act as intermediates have been isolated or detected in media fermented by normal or dried propionic acid bacteria. Lactic acid has been demonstrated by Foote and his associates[1] and by Fromageot and Tatum.[2] Virtanen and Karström found hexosemonophosphate.[3] Methylglyoxal was formed by the action of dried propionic acid bacteria on magnesium hexose phosphate[4]. Pyruvic acid was identified in the fermentation of glucose by *P. arabinosum.*[5] The same two men have isolated propionaldehyde from the fermentation of glycerol.[6] In 1936, Stone and Werkman isolated phosphoglyceric acid from a fermentation medium containing glucose, toluene, and sodium fluoride.

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[1] Foote, M., E. B. Fred, and W. H. Peterson, *Centr. Bakt. Parasitenk.*, Abt. II, **82**: 379 (1930).

[2] Fromageot, C., und E. L. Tatum, *Biochem. Zeit.*, **267**: 360 (1933).

[3] Virtanen and Karström, *loc. cit.*

[4] Pett, L. B., and A. M. Wynne, *Trans. Roy. Soc. Can.*, **27**: 119 (Sect. V) (1933).

[5] Wood, H. G., and C. H. Werkman, *Biochem. Jour.*, **28**: 745 (1934).

[6] Wood, H. G., and C. H. Werkman, *Proc. Soc. Exptl. Biol. Med.*, **31**: 938 (1934).

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CHAPTER XXIII

SOME MINOR FERMENTATIONS WITH INDUSTRIAL IMPLICATIONS

Our consideration of the possibilities of utilizing bacteria industrially would be unnecessarily incomplete if no mention was made of a number of processes, somewhat less sharply defined than those which have preceded, but which have well-grounded industrial implications. Through further research some of these minor fermentations may assume considerably greater significance, although they may not supply a basic reaction for an industry and may not yield specific end products that can be merchandized in pure form or in large quantity. Some of the bacterial processes here may prove to be contributory and useful as adjuncts in other industries. Others may aid the student to a somewhat clearer conception of processes long used in industry but in which the part played by microbic agencies has not been well defined. In such cases it is still impossible to draw conclusions or make statements that can be regarded as fixed and final.

Enzyme Production by Bacteria.—Active enzyme production is recognized as a qualification of many kinds of bacteria, but the field of industrial application has not been thoroughly investigated. As has been already pointed out in earlier chapters, bacteria produce several enzymes, including those which can hydrolyze carbohydrates, proteins, and fats and otherwise break down complex substances. The first step in the production of enzymes is to secure cultures of bacteria that will grow profusely and produce most efficiently the particular enzyme desired. Cultural conditions, such as the composition and pH of the medium, the use of sterile equipment and media, the temperature, the use of aeration, and other factors must be carefully worked out and controlled.

Bacterial enzymes, especially the amylases and proteases of *Bacillus subtilis* and its congeners, can be produced readily and may be used to considerable advantage in industry.

Production Method.—Wallerstein[1] has described a procedure for producing these bacterial enzymes, in particular, amylases. The medium used may contain starch or the products of an enzymatically digested starch; nitrogen from casein or from soybean or peanut cakes or other sources, hydrolyzed by means of enzymes or acids; mineral salts, such as

[1] Wallerstein, L., *Ind. Eng. Chem.*, **31**: 1218 (1939).

phosphates, and potassium, calcium, and magnesium salts plus traces of iron and manganese; and water. The medium, after filtration, sterilization, and cooling, is inoculated with a strain of *B. subtilis*. Best results are obtained when the inoculated medium is poured to form shallow layers in trays (contained in large culture vessels, each of which may have a capacity for as much as 1,000 gal.), when the incubation temperature is about 30°C., and when aeration is supplied. Temperature and aeration must be controlled carefully, the aeration being strongest at the beginning. Growth appears as bacterial films.

After the maximum enzyme content has been obtained, in perhaps a week, the culture medium containing the bacterial cells is centrifuged at approximately 14,000 r.p.m. to remove the bacteria. The resultant liquor is preserved by incubation at a low temperature or by the use of chemical antiseptics.

The enzyme-containing liquid may be used as such, or it may be concentrated and purified. Alcohol precipitation or salting-out methods may be used during the purification of the product. Possibly spray drying might also be employed in securing crude concentrates for some types of industrial use.

Alpha-amylase.—The alpha-amylase produced by *B. subtilis* has an optimum pH of 6.5 to 8,[1] is active at relatively high temperatures, 75 to 80°C. (even up to 95°C.), and may resist a short boiling in the presence of starch when the pH of the medium is 7 to 8. Alpha-amylase is the main amylase of bacteria. This enzyme is dextrinogenic.

Uses of Bacterial Enzymes.[1,2]—The amylases and proteases produced by bacteria may be used for the same general purposes as those produced by molds (see Chap. XXXII). Bacterial amylases may be used for the desizing of textiles; in the preparation of sizes for paper; for the production of starch-conversion products of low fermentability, which are occasionally used in the brewing industry; in the liquefaction of unmalted cereals; in the clarification of beer; in the preparation of chocolate sirups, in which case the chocolate starch is dextrinized and thus the sirup does not become thick; and for other purposes.

Bacterial proteases may be used for the unhairing and bating of hides; for the degumming of silk; for desizing acetate rayon when the size is made of gelatin or casein; for the separation of silver from photographic films by digestion of the gelatin of the film and liberation of the silver salts; and for other purposes.

The Fermentation of Cellulose.—A knowledge of cellulose fermentation is especially important to the soil microbiologist and to those inter-

[1] WALLERSTEIN, *loc. cit.*

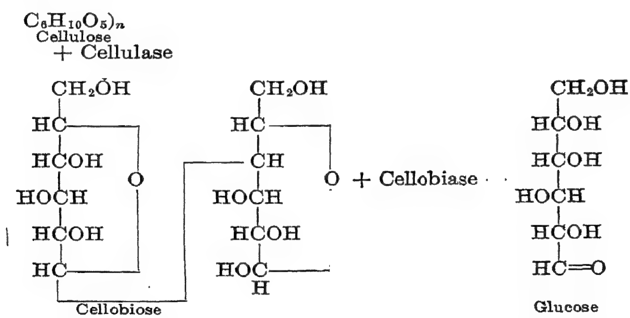
[2] WILLAMAN, J. J., Abstracts of Communications, Third International Congress of Microbiology, p. 335, New York, Sept. 2–9, 1939.

ested in the microbiology of textiles (Chap. XXXV) and wood (Chap. XXXVI). In view of the occurrence of such large quantities of cellulose in nature, and especially in the plant foods of animals, it is somewhat surprising to find that the enzyme "cellulase" appears to be absent in herbivores and other mammals. However, cellulose-fermenting bacteria are invariably found in the intestines of herbivores, and even, occasionally, in the intestines of human beings. Some types of cellulose can thus be made available as food for animals having a vegetarian diet.

Cellulose fermenters may be divided into two main groups, the aerobic organisms, including both bacteria and fungi, and the anaerobic or microaerophilic bacteria, which usually include the types found in the intestines. Bacterial types capable of attacking cellulose belong to six principal genera: *Cellulomonas*, small Gram-negative, nonsporeforming rods; *Cytophaga*, long flexuous rods; *Cellvibrio*, *Cellfalcicule*, *Clostridium*, and *Actinomyces*.

Cellulose-fermenting organisms are also found in the soil, in some surface waters, especially in the deposited material at mouths of slow streams, in fermenting manure, in compost heaps, in decaying sawdust, etc.

The breakdown of cellulose to glucose is the result of two enzymes: cellulase and cellobiase. Cellulase converts cellulose to cellobiose, while cellobiase converts cellobiose to glucose. This breakdown may be illustrated by the following scheme:[1]



The end products formed depend on the organisms and the conditions of the fermentation. Omeliansky worked many years ago with two sporeforming anaerobes. One of these produced principally fatty acids, carbon dioxide, and methane from cellulose, while the second produced fatty acids, carbon dioxide, and hydrogen. Khouvine reported the production of acetic and butyric acids, ethyl alcohol, carbon dioxide, and

[1] Salle, A. J., "Fundamental Principles of Bacteriology," McGraw-Hill Book Company, Inc., New York, 1939.

hydrogen by *B. cellulosae dissolvens* (*Cl. dissolvens*). This fermentation is a stage in the natural process by which methane is produced in muds and silt and in sewage sludges from cellulose.

Virtanen[1] has demonstrated that much of the cellulose of finely divided wood may be fermented by enriched cultures of thermophilic cellulose-fermenting bacteria. For example, 33.9 per cent of the cellulose found in birch dust was fermented in 10 to 14 days at 61°C. The amount of cellulose fermented is proportional to the fineness of the wood dust. Virtanen is of the opinion that cellulose is not chemically bound with lignin in wood.

Considerable research has been undertaken by Boruff, Buswell, Levine, and others concerning the utilization of cellulosic wastes for the production of fuel gas. Cellulose fermenters are important in these fermentations, which are anaerobic in nature.

For a further study of the fermentation of cellulose, it is suggested that the reader refer to some of the publications cited at the end of the chapter.

Microbiology of Leather Manufacture.—The essential steps in preparing fresh skins and hides for leather include preservation, soaking and fleshing, unhairing, "scudding," "bating," "drenching," pickling, and finally tanning and finishing. Obviously, there are many methods and modifications used. In several of these processes microbiological activities are implicit. The subject will be discussed very briefly.

Skins and hides as removed from animals are richly supplied with microbes, which if unchecked would rapidly injure or destroy the tissues. However, this activity can be largely prevented and the leather-making material may be preserved by salting (curing), by drying, by combining drying and salting, by pickling, or by use of disinfectants. The first of these methods is most commonly practiced.

Soaking is carried out to remove blood, dirt and manure, salt, and other soluble material; and at the same time to "plump," or swell, and to soften the skins and hides by absorption of water. During soaking there is danger of undesirable bacterial action unless controlled. By keeping the soak water cool (*i.e.*, below optimum temperature) and by changing it several times, bacterial growth may be somewhat inhibited.

In the so-called "fleshing" of hides, adipose tissue and other undesirable portions are trimmed from the hides.

Various methods are employed for unhairing the skin or hide. In the oldest method, the hides were placed in a sweating chamber, where the humidity was high and the atmosphere warm. Bacteria inherent on the surface, especially skin cocci, caused an incipient putrefaction and dis-

[1] VIRTANEN, A. I., Abstracts of Communications, Third International Congress of Microbiology, p. 333, New York, Sept. 2–9, 1939.

solution of some of the cementing material at the roots of the hair. This process was commonly employed with sheepskins, and the fleece could then be "pulled" in intact form. Sweating processes could be controlled only with difficulty, and consequently the hides or skins were injured, many of them severely. Lime pits or saturated lime water baths have been more commonly used in unhairing processes, the skins being swelled and hair sheaths dissolved at a pH of about 12.5, according to Wilson.

Limewater containing sodium or potassium sulphite or other depilatory compounds, a sodium sulphide and lime paste, alkalies, acids or enzymes, which may include pancreatin solutions, or bacterial or mold proteases, have been used at various periods in unhairing processes. The most recent trend is toward the use of enzymes.

The loosened hair, sebaceous and sudoriferous glands, dirt and the lime soaps formed, and other material (not a part of the true skin) are removed by scudding, or scraping with a tool comparable to a large knife or drawshave with a smooth but dull edge. The skins or hides that have been limed contain lime salts that must be removed. It is also important to remove all other material except the collagen fibers that make up the firm tissues of the different layers of the skin. This removal is accomplished by bating.

Bating is an enzymic process for bringing about physical and chemical changes in the hides or skins, in which neutral salts may or may not be used. The oldest methods consisted in using infusions of dog manure, known as "puring," or of pigeon or fowl manure, known as "bating." It was shown by Wood that the effective action of the dung was due to the enzmes contained in it—lipase, rennet, and peptic, tryptic, and amylolytic enzymes. Later pancreatic enzymes or other enzyme mixtures were substituted for the dung bates. In this process, the pH is usually slightly on the alkaline side of neutrality.

During bating, the enzymes cause the removal of the coagulated or coagulable proteins of the hide, the dissolved keratin, and reticular tissue. The skins are partially delimed, grain roughness may be removed to some extent, and the stretching ability of the elastin fibers of the skin is restored.[1] The last three of these changes may be brought about by the use of lactic acid or ammonium chloride. The pH is likewise adjusted during bating.

The skins of sheep, goats, and calves are sometimes "drenched" in a bran infusion. Bacteria ferment the carbohydrates of the bran with the production of organic acids, especially lactic, and gases. Drenching is effective because the lactic acid forms easily soluble lime salts that can

[1] McLaughlin, G. D., J. H. Highberger, F. O'Flaherty, and K. Moore, *Jour. Am. Leather Chem. Assoc.*, **24**: 339–379 (1929).

be washed out and thus prepares the skins for the eventual process of tanning. The hides may be pickled in a bath containing dilute sulphuric acid and sodium chloride previous to tanning.

The Curing of Tobacco.—Tobacco undergoes a series of curing treatments before it is manufactured, in order to improve its texture and aroma. Whether these changes are to any marked extent the results of specific bacterial fermentation has been a subject of discussion for many years. It is now generally believed, as a result of the evidence accumulated over many years, that the principal changes taking place during the curing process are largely the result of the action of the enzymes, especially the oxidases, present in the tobacco leaves and not dependent on bacterial action. Nevertheless, the changes induced by the large number of bacteria present might easily serve as a contributory factor in the whole process. A few earlier studies indicated that the finer qualities of tobacco were the results of the action of specific types of bacteria. This seems never to have been confirmed by carefully controlled and adequate research.

After harvesting, the tobacco leaves are dried, piled in heaps, moistened (sometimes with dilute solutions of sugars, malt extract, honey, or other sirups) and permitted to undergo spontaneous fermentation or curing. During this process heat is evolved and the temperature of the mass may rise to 55 to 60°C. The physical appearance and chemical structure of components of the leaves are changed. Aroma and flavor are developed. Starch and reducing sugars tend to disappear. The quantities of malic acid, nicotine, pentosans, and protein decrease, while there is an increase in the quantity of citric acid. Carbon dioxide and ammonia are evolved. Hydrolytic, oxidative, proteolytic, and other types of enzymes are apparently concerned in the curing process.

As would be expected from the mode of cultivation the flora on the leaves may be quite varied. According to a summary of the many bacterial studies prepared by Salle,[1] *Bacillus subtilis*, *B. mycoides*, *B. polymyxa*, *Proteus vulgaris*, and species of the genus *Aspergillus* and other molds are present on tobacco leaves. This list is probably incomplete. Giovannozzi states that the unfermented leaf of Kentucky tobacco may contain from 100,000 to 100,000,000 bacteria per gram (dry weight). Blastomycetes also may develop during the first part of the fermentation, but may disappear later. Cocci are likewise present in fermented tobacco, frequently in larger numbers than the bacilli.[2]

This fermentation, like that of silage, grass, and other materials in which components of living leaf tissues and the adherent microbes may

[1] SALLE, *loc. cit.*

[2] GIOVANNOZZI, M., *Chimica e industria (Italy)*, **40**: 768 (1937). (*Chem. Abstract.*)

both function, will continue to offer an inviting field for microbiological research.

Ethanol Production by Bacteria.—Ethanol is produced in varying quantities by a relatively large number of bacteria. Usually the ethanol is produced in small amounts only and is one of several products, thus making industrial production unfeasible. Research may somewhat change this viewpoint, but bacteria are not likely to be serious competitors of yeast in the near future.

A medium containing 2 per cent glucose and yeast extract was fermented by *Termobacterium mobile* Lindner (*Lactobacillus mobile*) with the production of 45.2 per cent of ethanol, 45.1 per cent of carbon dioxide, and 7.2 per cent of lactic acid.[1] A fermentation using this organism has been carried out on an industrial scale in Germany. The organism was isolated in Mexico by Lindner from the fermenting juice of a large cactus. This fermented juice, locally used as an alcoholic beverage, is called "pulque."

Weizmann[2] has reported a yield of 25.6 per cent of ethanol from a sucrose solution by *Clostridium ethylicum* (Weizmann). From 720 g. of cane sugar, 24 g. of volatile acids, as butyric acid, were formed. The organism was isolated from *Hibiscus sabdariffa*.

Bacillus asiaticus mobilis Castellani, an organism believed to be closely related to *Escherichia coli*, gives rise to ethanol, hydrogen, and butylene glycol.

Sarcina ventriculi[3] Goodsir forms ethanol, carbon dioxide, and acetic acid as the principal products from glucose. In one experiment reported by Kluyver, 43.7 per cent of the glucose was fermented to ethanol. *Sar. ventriculi* is aerobic[4] and has an optimum temperature of 30 to 35°C.

Ethanol is produced by certain acetic acid bacteria, for example, *Acetobacter ascendens*, *A. suboxydans*, and *A. pasteurianum*, under anaerobic conditions.[5]

It is produced in small quantities by heterofermentative lactic acid bacteria, by certain cellulose-fermenting bacteria, and by the bacteria that produce butanol and acetone.

Acetylmethylcarbinol, 2,3-Butylene Glycol and Diacetyl.—Acetylmethylcarbinol and 2,3-butylene glycol are frequently found together in

P., "Mikroskopische und biologische Betriebskontrolle in den Gärungsgewerben, . . . " Paul Parey, Berlin, 1930.

[2] WEIZMANN, C., *Jour. Soc. Chem. Ind.*, **57**: 1049 (1938).

[3] SMIT, J., "Die Gärungssarcinen," Gustav Fischer, Jena, 1930; KLUYVER, A. J., "The Chemical Activities of Micro-organisms," University of London Press, Ltd., London, 1931.

[4] KLUYVER, *loc. cit.*

[5] BUTLIN, K. R., "The Biochemical Activities of the Acetic Acid Bacteria," Chemistry Research, Special Report 2, H. M. Stationery Office, London, 1936.

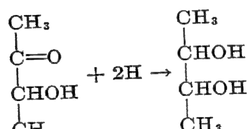
certain fermentations. They are produced, together with ethyl alcohol, by *Aerobacillus polymyxa* (*B. polymyxa*), according to Donker.[1] Some anaerobes, believed to be related to *A. polymyxa*, have been studied by Langlykke, Peterson, and McCoy. Table 65 presents some data concerning the fermentation of glucose by these organisms (the medium used was described on page 230).

TABLE 65.—THE PRODUCTION OF BUTYLENE GLYCOL AND OTHER PRODUCTS FROM GLUCOSE[1]

			Products (based on glucose)			
Culture no.	Glucose fermented, per cent	Acidity 0.1 N NaOH in 10 cc., cc.	Butylene glycol, per cent	Acetyl-methyl-carbinol, per cent	Ethyl alcohol, per cent	Total, per cent
53	89.3	2.30	6.0	3.8	14.7	24.5
56	89.7	2.35	7.1	6.9	18.0	32.0

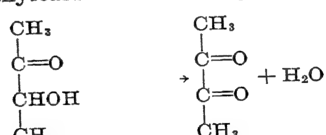
[1] LANGLYKKE, A. F., W. H. PETERSON, and E. McCOY, *Jour. Bact.*, **29**: 333 (1935)

Some bacteria have the ability to reduce acetylmethylcarbinol to 2,3-butylene glycol, a change that may be indicated as follows:



Acetylmethylcarbinol 2,3-Butylene glycol

Other bacteria are capable of oxidizing 2,3-butylene glycol to acetylmethylcarbinol. One such organism is *A. aerogenes*. Still other bacteria may oxidize acetylmethylcarbinol to diacetyl:



Acetylmethylcarbinol Diacetyl

Diacetyl is important as an aroma-producing component of butter. Butter cultures of the desired type always contain diacetyl and acetylmethylcarbinol in relatively large amounts. The latter substance is oxidized to diacetyl in butter cultures by such citric acid fermenting organisms as *Streptococcus citrovorus* and *Strept. paracitrovorus*, according

[1] DONKER, H. J. L., Thesis, Technische Hoogeschool, Delft, 1926.

to Michaelian and Hammer.[1] The production of acetylmethylcarbinol and especially diacetyl by butter cultures or pure cultures of the citric-acid-fermenting streptococci is favored by the acidification of the cultures with a mixture of citric and sulphuric acids and the presence of an abundant supply of oxygen. Obviously the reductions of diacetyl and acetylmethylcarbinol to 2,3-butylene glycol cause a diminution in the amount of the aroma-producing substance, diacetyl.

The Fermentation of Rhamnose by Bacterium rhamnosifermentans.[2] The fermentation of rhamnose ($CH_3 \cdot CHOH \cdot CHOH \cdot CHOH \cdot CHOH \cdot CHO$) by *Bacterium rhamnosifermentans* is of particular interest, because propylene glycol ($CH_3 \cdot CHOH \cdot CH_2OH$) is formed as one of the end products.

TABLE 66.—QUANTITATIVE RESULTS OF THE FERMENTATION OF RHAMNOSE BY *Bact. rhamnosifermentans*[1]

	Weight, grams	Per cent carbon	Millimols	Millimols per 100 millimols of fermented rhamnose	Milliequivalents of available hydrogen	Available hydrogen, per cent
Rhamnose:						
Added	9.956					
Recovered	2.737					
Fermented	7.210	100	44	100	2,600	100
Carbon dioxide	0.623	5.36	14	31.8		
Hydrogen	0.032	......	14	31.8	63.6	2.4
Formic acid	0.057	0.50	1	2.3	4.6	0.2
Acetic acid	1.935	24.42	32	73.0	584.0	22.5
Ethanol	0.056	1.00	1	2.3	27.6	1.1
Succinic acid	1.752	22.50	15	34.0	476.0	18.3
Propylene glycol	3.344*	50.00	44	100	1,600	61.0
Total	......	103.78	..	.....	....	105.5

* Not determined, but computed.

[1] KLUYVER, A. J., und CH. SCHNELLEN, *Enzymologia*, **4**: 7–12 (1937).

The organism was described by Castellani[3] as *Bacillus rhamnosifermentans*. It is a Gram-negative, facultative aerobe, which shows selective action in respect to the carbohydrates fermented and the nitrogen compounds utilized. Rhamnose in peptone water (1 per cent) containing 1.5 per cent of calcium carbonate was fermented nearly completely in 10 days by *Bact. rhamnosifermentans*, and more vigorously

[1] MICHAELIAN, M. B., and B. W. HAMMER, *Iowa Agr. Expt. Sta., Research Bull.* 205, 1936.

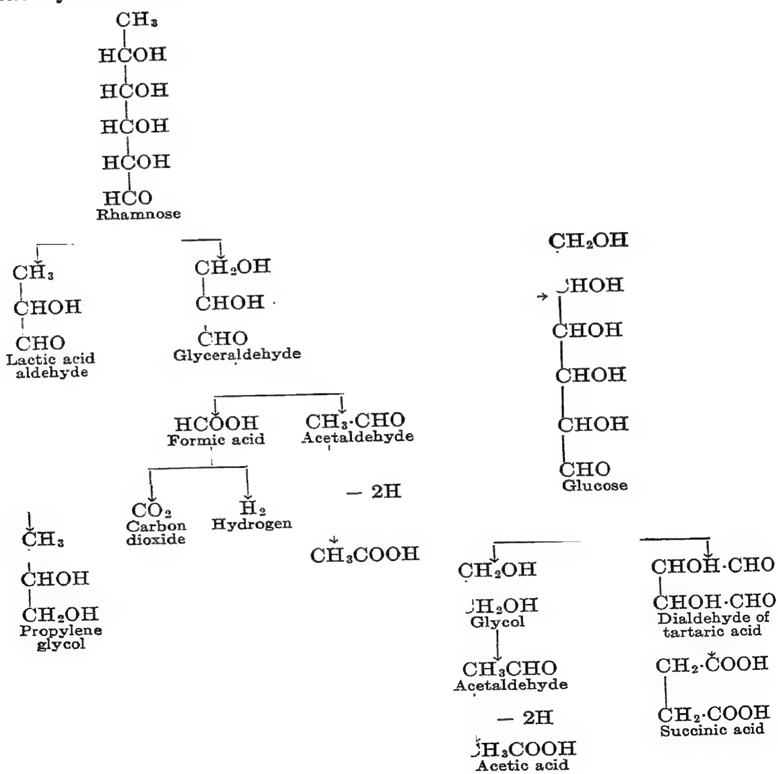
[2] KLUYVER, A. J., und CH. SCHNELLEN, *Enzymologia*, **4**: 7–12 (1937).

[3] CASTELLANI, A., *Ann. Inst. Pasteur*, **47**: 297–305 (1931).

than any other carbohydrate investigated.[1] Yeast water was unfavorable as a nitrogen source. Equimolar quantities of hydrogen and carbon dioxide gases were produced by *Bact. rhamnosifermentans*, which is believed to be closely related to *Escherichia coli*.

Table 66 shows the products identified and quantitatively estimated in the fermentation of a medium, which contained 1.5 per cent rhamnose and 1.5 per cent calcium carbonate in peptone water. The amount of propylene glycol represented was obtained by calculation and not by quantitative estimation of its content in the fermentation medium.

On the basis of the available facts and the assumption concerning propylene glycol, Kluyver and Schnellen have suggested the following scheme to indicate the course of the fermentation of rhamnose by *Bact. rhamnosifermentans*:



[1] KLUYVER und SCHNELLEN, *loc. cit.*

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CHAPTER XXIV

THE MOLDS

True molds are members of the division of the plant kingdom known as *Thallophyta*. They do not possess chlorophyll nor are they differentiated into leaves, stems, or true roots. They are widely distributed, especially in the soil.

The role of molds in nature is of very great importance to man. Pathogenic molds are the causes of diseases in plants and animals. Nonpathogenic species include those involved in the breakdown of organic matter in the soil; those concerned in the deterioration and destruction of timber, textiles, foods, and other products; and those with distinct industrial importance, as the molds concerned with the ripening of cheese and the production of commercially valuable organic acids, enzyme preparations, sauces, and related products.

True molds may be divided into four main classes: the *Phycomycetes*, which usually possess nonseptate mycelia; the *Basidiomycetes*, which possess septate mycelia and produce sexual spores exogenously on stalks; the *Ascomycetes*, which possess septate mycelia and produce sexual spores endogenously in sacs; and the *Fungi Imperfecti*, which possess septate mycelia but often produce no sexual spores[1] so far as is now known. (Refer to Fig. 35 for subdivisions of these classes.)

General Description.—The individual mold, structurally, may be considered to be made up principally of mycelium and spores. The mycelium is a collection or aggregate of hyphae (singular, hypha), which are thread-like filaments of protoplasm. Hyphae are of two main types: fertile hyphae, which are concerned with the production of reproductive cells or fruit bodies, the spores; and vegetative hyphae, the function of which is to secure nutrient substances from the substrates.

Hyphae may be septate or nonseptate. Septate hyphae are hyphae containing crosswalls or septa, which divide the mold into cells. Nonseptate hyphae contain no crosswalls, but are multinucleate. Molds of the latter type are coenocytic. Cells of septate molds may contain only one nucleus, as in the case of the *Ascomycetes*, or two nuclei, as in the *Basidiomycetes*.

[1] Henrici, A. T. "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., New York, 1930.

FIG. 35.—A CLASSIFICATION OF THE FUNGI[1]

Classes — Subclasses — Orders — Families — Genera

- *Thallophyta*
  - Algae
  - Lichens
  - Fungi
    - *Myxomycetes* (slime molds)
    - *Schizomycetes* (bacteria)
    - *Eumycetes* (true molds)
      - *Phycomycetes*
        - *Zygomycetes*
          - *Mucorales*
            - *Mucoraceae*
              - *Mucor*
              - *Rhizopus*
              - *Zygorhynchus*
              - etc.
            - *Thamnidiaceae*
              - *Thamnidium*
              - etc.
            - etc.
          - *Entomophthorales*
          - etc.
        - *Oömycetes*
        - *Archimycetes*
      - *Ascomycetes*
        - *Plectomycetes*
          - *Plectascales*
            - *Byssochlamys*
            - *Endomyces*
            - *Monascus*
            - *Saccharomyces*
            - etc.
          - *Erysiphales*
          - *Exoascales*
        - *Discomycetes*
        - *Pyrenomycetes*
      - *Basidiomycetes*
        - *Hemibasidiomycetes*
        - *Protobasidiomycetes*
        - *Autobasidiomycetes*
      - *Fungi imperfecti*
        - *Hyphomycetales*
          - *Mucedinaceae*
            - *Aspergillus*
            - *Botrytis*
            - *Monilia*
            - *Oöspora*
            - *Penicillium*
            - *Scopulariopsis*
            - etc.
          - *Dematiaceae*
            - *Alternaria*
            - *Cladosporium*
            - *Helminthosporium*
            - *Hormodendrum*
            - *Stemphylium*
            - etc.
          - etc.
        - *Melanconiales*
        - *Sphaeropsidales*

[1] HENRICI, A. T., "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., New York, 1930; SMITH, G., "An Introduction to Industrial Mycology," Edward Arnold & Co., Ltd., London, 1938; FITZPATRICK, H. M., "The Lower Fungi—Phycomycetes," McGraw-Hill Book Company, Inc., New York, 1930.

Molds increase in mass or grow by the extension of the tip cells (apical growth) or in septate types by apical growth and by a division of the cells in any part of the hypha (intercalary growth).

The young cells of a mold are usually filled with dense cytoplasm, but old cells contain many vacuoles and reserve food materials, such as fat globules and glycogen. The cell wall is believed to be composed of chitin.

Spores may be asexual or sexual in nature. Asexual spores may be formed within a closed vessel or spore case known as a "sporangium" (*angium*, "case"), which is supported by a sporangiophore (*phore*, "bearer") in the case of the genera *Mucor* and *Rhizopus*, members of the class *Phycomycetes*, or they may arise from special hyphae, known as "conidiophores." In the latter case, the spores are known as "conidia."

Asexual spores are borne in various other ways. The reader interested in this aspect of mycology is referred to some of the texts listed at the end of this chapter.

The chlamydospore is a spore with thickened walls and generally is derived from a vegetative cell.

Ascospores are sexual spores and are produced characteristically in a specialized sac or ascus by the class *Ascomycetes*.

Zygospores (*zygo*, "yoke") are spores with thick walls formed as the result of the conjugation of two terminal hyphae arising from different colonies, these colonies representing plus and minus strains of the species. Zygospores are thus sexually produced spores.

Distinction is made between molds and a vast number of fungi that are the causative agents of plant diseases and thus live an essentially parasitic life. From the standpoint of industrial microbiology, the term "mold" is generally given to aerobic saprophytes that grow on organic matter or solutions with the formation of expansive masses of mycelium, which may be thin and superficial in character or which may occur as felted masses of tough and/or semigelatinous nature. The mycelia can penetrate the substrate for some distance, especially when growing on cellular tissues or amorphous masses of material.

The number of known species of molds is large, but as their classification is often difficult because of great differences in appearance on different substrates it is unwise to attempt exact figures.

Molds are especially characterized by the ability to elaborate a great variety of enzymes, and this physiological qualification undoubtedly accounts for their ability to thrive on so many materials and in the presence of very small amounts of organic matter. Enzyme production by certain species will be considered more fully in a later section of this chapter.

The Growth Requirements of Molds.—Certain elements are essential for the growth of molds, such as nitrogen, carbon, hydrogen, oxygen,

sulphur, potassium, phosphorus, magnesium, and other elements. Some molds require special organic substances, such as thiamin, for growth.

Nitrogen Sources.—Molds, in general, may utilize a large number of nitrogen-containing compounds. These differ in relative value, some stimulating growth by yielding nutritive substances, and some being especially favored on account of their effect in producing large yields of desired end products. The type of nitrogen compound selected is thus of much importance to the fermentologist, who is not only interested in the yield but also in the recovery of the end product in as pure a condition and as free from color as possible.

In general, ammonium salts, nitrates, proteins, peptones, amino acids, and urea are satisfactory sources of nitrogen.

Carbon Sources.—Molds obtain their energy essentially from carbon-containing compounds. In the complete breakdown of a carbohydrate to carbon dioxide and water, as in aerobic respiration, a relatively large amount of energy is liberated. In the anaerobic or partial anaerobic breakdown of carbohydrates, intermediate products are formed which possess less energy value than the original carbohydrate. The energy thus liberated is less than that evolved during aerobic respiration. This type of energy reaction is the one commonly associated with fermentation.

Many carbon-containing compounds have been examined as sources of carbon by molds. For example, *Aspergillus oryzae*[1] utilized 51 compounds, principally alcohols and acids, for growth and respiration. Olive, linseed, and walnut oils; triolein; pentosans; amylopectin; cellulose; some higher paraffins[2]; starches; sugars; and other compounds have been utilized by different molds, in addition to alcohols and acids.

Other Elements.—Iron, zinc, copper, manganese, molybdenum, and gallium appear to be important elements for the growth of certain molds, in particular, *Aspergillus niger*.[3] These elements constitute some of the "trace elements."

Growth Media.—Most molds grow well in a medium containing an appropriate starch or sugar, a usable source of nitrogen, and salts supplying essential mineral elements. An acid reaction is desirable.

Media may be classified as synthetic and natural media. Natural media are obviously the tissues or juices of plants or animals in their native state. It is highly desirable, however, especially for identification purposes, to cultivate molds in media that can be duplicated exactly at any time or in different parts of the world. Such synthetic media are prepared with pure sugars and chemically pure inorganic or organic com-

[1] TAMIYA, H., *Acta Phytochim. Japan*, **6**: 1 (1932).

[2] BIRKINSHAW, J. H., *Biol. Rev., Cambridge Phil. Soc.*, **12**: 357 (1937).

[3] STEINBERG, R. A., *Bull. Torrey Botan. Club*, **61**: 241 (1934); **62**: 81 (1935). *Jour. Agr. Research*, **51**: 413 (1935); **55**: 891 (1937); etc.; BIRKINSHAW, *loc. cit.*

pounds. Steinberg[1] has recently reviewed the subject of the growth of fungi in synthetic nutrient solutions. This excellent paper contains many references to the nutrition of fungi.

Raulin's Medium.[2]—One of the oldest synthetic media is that of Raulin, which had the following unduly complex composition:

	Grams
Water	1,500
Sucrose	70.0
Ammonium nitrate	4.0
Tartaric acid	4.0
Ammonium phosphate	0.6
Potassium carbonate	0.6
Magnesium carbonate	0.4
Ammonium sulphate	0.25
Zinc sulphate	0.07
Ferrous sulphate	0.07
Potassium silicate	0.07

The foregoing medium has a highly acid reaction (the pH is approximately 2.9).[2]

Czapek's Medium.[3]—For growth and isolation of molds Czapek's medium is widely used. This has the following composition, as modified by Dox and Thom:

	Grams
Sucrose	30.0
Sodium nitrate ($NaNO_3$)	2.0
Potassium phosphate (K_2HPO_4)	1.0
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.5
Potassium chloride (KCl)	0.5
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0.01 or trace
Agar	15 (12 to 20)
Water	1,000 cc.

The final reaction of the preceding medium is neutral or slightly[2] acid. By using potassium dihydrogen phosphate (KH_2PO_4) instead of potassium monohydrogen phosphate (K_2HPO_4) a definitely acid reaction is obtained. This is preferred by some workers.

Glucose or other sugars, in varying quantities, may be substituted for sucrose in the foregoing formula, thus obtaining a medium with wider applications. Mucors do not grow well on Czapek's medium containing sucrose, for they do not readily utilize this sugar.

[1] STEINBERG, R. A., *Botan. Rev.*, **5**: 327 (1939).

[2] SMITH, G., "An Introduction to Industrial Mycology," Edward Arnold & Co., London, 1938.

[3] THOM, C., and M. B. CHURCH, "The Aspergilli," The Williams & Wilkins Company, Baltimore, 1926.

In order to avoid a browning of the medium or the production of turbidity, the phosphates should be dissolved separately in a small portion of the water, sterilized, and added to the main portion of the sterilized medium.

Malt Medium.—A medium, liquid or solid, prepared from malt extract is useful where the cultivation of molds and yeasts is concerned. Such a medium has been recommended for the determination of molds and yeasts in butter.

One malt-extract agar is prepared by dissolving 30 g. of dehydrated malt extract (Difco) and 15 g. of agar (Bacto) in 1,000 cc. of distilled water and autoclaving at 15 lb. pressure for 20 min. The final reaction is 5.5.[1]

Sabouraud's Medium.—Parasitic molds grow well in a medium that is a modification of the original formula of Sabouraud.[2] Sabouraud's dextrose agar is prepared by dissolving 10 g. of peptone, 40 g. of dextrose, and 15 g. of agar (Bacto) in 1,000 cc. of distilled water and autoclaving the medium at 15 lb. pressure for 20 min. The final reaction is 5.6.[1]

Sabouraud's maltose agar is prepared in the same manner except that maltose is used instead of dextrose. Maltose is more satisfactory for the cultivation of certain pathogenic molds than dextrose, for example, of *Microsporon audouini* and of *M. lanosum.*

Liquid media are prepared by omitting the agar. Such media are very useful in certain types of work with molds.

Natural Media.—Prune, potato, carrot, bean, wort, and other juices or extracts with or without agar are sometimes useful in the cultivation of molds.

Nutrient agar or gelatin, such as is used for the growth of bacteria, may be enriched with carbohydrates and adjusted to an acid reaction.

For further data concerning media, the reader is referred to standard texts and publications on mycology and to the reference below.[3]

Methods of Isolating Molds.—There are several methods for isolating molds in pure culture. Some of these are similar to methods used in isolating bacteria or yeasts.

By Agar Dilution.—A series of bacterial culture tubes, perhaps four to six, each containing about 10 cc. of a suitable agar medium, is heated in a water bath to melt the agar. The contents of the tubes are cooled to 44 to 42°C. and maintained at that temperature in a water bath until the tubes are used. At this temperature the agar will not solidify, nor

[1] "Difco Manual," Difco Laboratories, Inc., Detroit, 1938.

[2] Henrici, *op. cit.*

[3] Levine, M., and H. W. Schoenlein, "A Compilation of Culture Media for the Cultivation of Micro-organisms," The Williams & Wilkins Company, Baltimore, 1930.

will it cause injury to the mold. A small amount of the mold-containing material is added to the first of the series of tubes. It is mixed thoroughly with the agar by agitation and a small loopful of this seeded agar is transferred to the second tube of the series. The first tube of agar is then poured aseptically into a petri dish. The contents of the second tube are shaken carefully, one loopful being then transferred to the third tube and the rest poured into a petri dish. This process continues until 4 to 6 or more plates have been poured. The culture is diluted by this method, and at least one of the plates should contain the desired mold in such dilution that a pure culture may be secured. Agar slopes (slants) may be inoculated from this colony.

An alternate method is similar to the above in that tubes of agar are melted and cooled to 44 to 42°C. The culture is added to the first tube and after the tube is shaken carefully, the contents are plated. The second tube of agar is then added to the first tube, which contains a few mold spores in the agar still adhering to the sides and bottom of the tube. The latter tube is shaken and the contents poured into a petri dish. A third, fourth, fifth, and perhaps other tubes of agar are added successively to the first tube in the same fashion, and the agar is poured.

By Picking Spores from a Single Spore Head.—In this method, the mycologist selects a colony of the mold that he believes is a pure culture and using a hand lens or the low power of the microscope picks mold spores from a single spore head with a sterile needle and transfers them to a tube containing a medium favorable for growth.

If the plate from which the mold colony is selected contains other types of molds, there is a possibility of obtaining a mixed culture.

By the Micromanipulator.—This method, though practicable, requires a certain amount of experience. Excellent results may be obtained by the use of this method.

By the Germination of a Single Spore.—A dilution of spores is made in sterile water or saline until a drop contains just one spore. (Ascertained by an examination of droplets on a slide on the stage of a microscope.) Droplets are then placed in isolated portions on the surface of agar, their position being marked in order to locate the correct culture in case the plate contains a contaminant.

By a Modification of the Keitt Single-spore Method.—In Ezekial's[1] modification of the Keitt single-spore method,[2] a nutrient agar, selected for growing the desired mold, is melted, poured into a petri dish to form a thin layer and permitted to solidify. Using a needle (with a spatulate tip) infected with the spore-containing material, which has been diluted properly, four to five parallel streaks are made on the surface of the agar.

[1] Ezekial, W. N., *Phytopathology*, **20**: 583 (1930).

[2] Keitt, G. W., *Phytopathology*, **5**: 266 (1915).

The dish is then inverted, incubated for 16 to 24 hr., and examined through the bottom with the aid of the 16-mm. objective of a microscope in order to locate a sporeling. When a sporeling has been detected, its position is marked with ink on the plate and it is examined more thoroughly with a high magnification of the microscope. Using a needle with a cylindric tip, a disk of agar containing the sporeling is cut out, placed on a thin portion of the agar on an agar slope and again examined microscopically to make sure that only one sporeling is present.

By the Hansen Method.[1]—In another method a dilute suspension of spores is prepared in agar, the agar then being sucked into capillary tubes, the diameters of which are not much greater than the diameters of the spores. The capillary tubes are examined under the microscope. When an isolated spore is found the tube is broken in such a manner that the segment contains a single spore. The glass is treated with alcohol and then placed into fresh medium. Growth emerges from the tube, and a colony develops. This method operates successfully with large, colored spores but not well with small spores.

Single-spore isolations may be made also by the Hansen method or by the Lindner method (refer to Chap. II).

The Identification of a Mold.—The basis for the identification of a mold is an accurate and complete description of the organism. Information obtained by the use of the naked eyes, the hand lens, and the microscope, in the manner outlined in the following paragraphs, is usually sufficient.

It is desirable to cultivate the mold on different types of solid media in order to ascertain the medium best adapted to its growth. Ordinarily Czapek's medium is selected to study the characteristics of a mold that may be used for industrial purposes. This medium has been used in studying the aspergilli and the penicillia by Thom, Church, and others; in studying the *Actinomycetes* by Waksman; and in studying other molds. A large number of molds grow satisfactorily on this medium. For the sake of comparative purposes it is a good plan to use this medium, then, if the mold grows well on it.

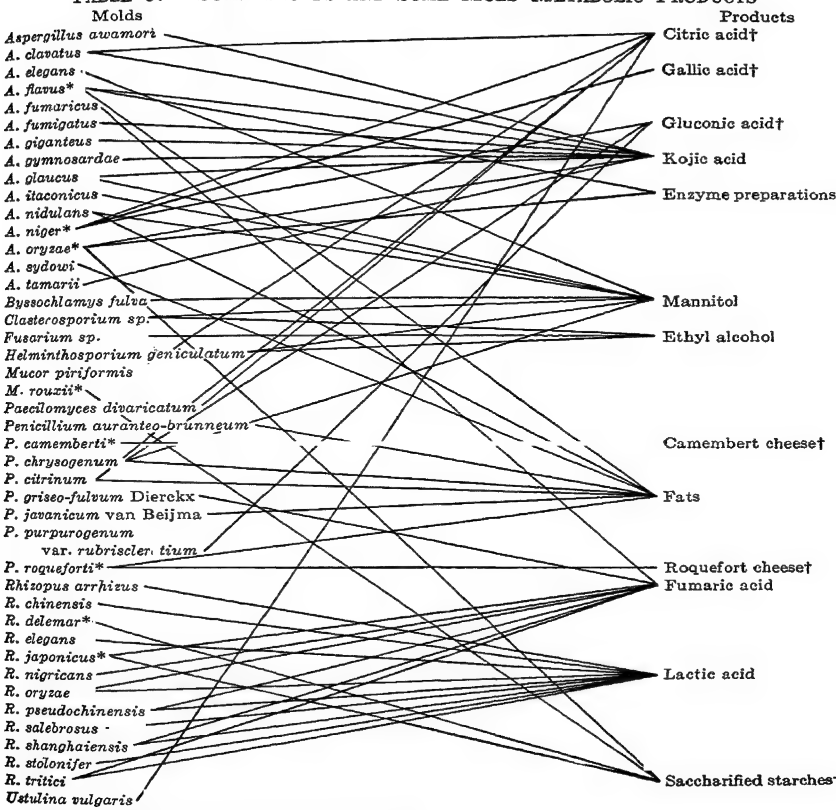
Individual colonies on Czapek's agar or other medium may be studied with the naked eyes, the hand lens, and the low magnification of the microscope. The following information may be obtained from such observations: the rate of growth; the appearance or growth habit; the nature, arrangement, size, and color of the fruiting bodies and hyphae; the elevation and density of different parts of the colony; the presence or absence of perithecia; variation in the shape and sizes of the mold heads; and other data.

[1] HANSEN, H. N., *Science*, **64**: 384 (1926).

The plate may be turned over and the color of the underside of the colony observed, also any coloration produced in the medium.

The information gained from the foregoing study may be sufficient to identify the mold insofar as the class or order is concerned, but further study with the aid of the oil-immersion lens of the microscope is usually necessary in order to obtain enough information to identify the mold as to genus and species.

TABLE 67.—SOME MOLDS AND SOME MOLD METABOLIC PRODUCTS*



* Mold species of present industrial importance.
† Products produced on an industrial basis by mold action.

Slide cell cultures are very helpful in studies involving the minute structure of molds. Such cultures may be examined either stained or unstained. The following observations are made on the spor

shape, size (average and extremes), color, markings, and arrangements. Fertile hyphae are examined for branching, septation, width, color, markings, and the nature of the walls, whether smooth, pitted, or warted. Crystals of chemical compounds or juices elaborated by the mold should be observed.

On the basis of the descriptions thus obtained, an attempt may be made to identify the mold, using a text which describes the genera.

Important Genera of Molds.—From an industrial standpoint, species of the genera *Aspergillus*, *Penicillium*, *Rhizopus*, and *Mucor* are by far the most important at the present time. Table 67 summarizes some of the products manufactured on a large scale by the action of molds. It will be noticed that *Aspergillus niger* is particularly important, strains of this organism being used in three important industrial fermentations —the citric, gallic, and gluconic acid fermentations. From amongst the penicillia are species which are important in the ripening of cheeses and which may be used to produce gluconic and citric acids. Species of the genus *Rhizopus* and the genus *Mucor* are of importance in the saccharification of starchy materials. Recent investigations (see Chap. XXVII) have indicated that certain species of *Rhizopus* may soon become valuable in the industrial production of *d*-lactic acid.

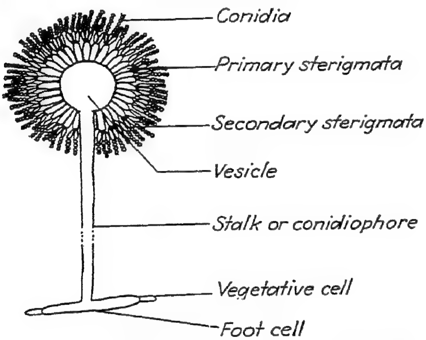


FIG. 36.—Diagram of *Aspergillus*. (*Adapted from Thom and Church, "The Aspergilli," The Williams & Wilkins Company, Baltimore*, 1926.)

Products Formed by Molds.—Table 68 lists some of the products formed by molds. Many of these are of a complex nature.

Characteristics of the Genus Aspergillus.—The mycelium consists of septate branching hyphae, which may be brightly colored or colorless and which may produce crusts or sclerotia.[1] The mycelium is usually partially submerged in the substrate and partially aerial.

The foot cell is a specialized, enlarged, thick-walled cell that gives rise to a fertile hypha, *i.e.*, the stalk or conidiophore. The foot cell is usually, but not always, submerged in the substrate.

The stalk, or conidiophore, arises approximately perpendicularly to the long axis of the foot cell. Its walls may be smooth, pitted, or rough. It may be septate or unseptate. At the apex, the stalk usually enlarges to form a vesicle.

[1] THOM and CHURCH, *op. cit.*

The vesicle, which supports the sterigmata, is globose, hemispherical, elliptical, clavate, calyptrate, or of other shape. A portion or all of its surface is covered with sterigmata.

TABLE 68.—SOME PRODUCTS FORMED BY MOLDS

Acids	Alcohols	Enzymes	Pigments	Polysaccharides	Sterols	Miscellaneous
Acetic	*i*-Erythritol	Amidase	Aspergillin	Capreolinose	Cholesterol	Acetaldehyde
Aconitic	Ethyl	Amylase (α & β)	Aurantin	Galactocarolose	Ergosterol	Alboleersin
Allantoic	Glycerol	Carboxypolypeptidase	Aurofusarin	Glycogen	Fungisterol	Dimethyl selenide
Byssochlamic	Mannitol	Catalase	Auroglaucin	Luteic acid	Phytosterol	Ergot
Carlic		Cellobiase	Boletol	Mannocarolose		Ergosteryl palmitate
Carlosic		Cytase	β-Carotene	Mycodextrin		Ethyl acetate
Carolic		Dextrinase	Catenarin	Rugulose		Gums
Carolinic		Dipeptidase	Chrysogenin	Starch (mold)		Hydroxylamine
Citric		Emulsin	Citrinin	Trehalose		Lipins
3:5-Dihydroxyphthalic		Erepsin	Citromycetin	Varianose		Luteoleersin
Dimethylpyruvic		Gentianase	Cynodontin			Palitantin
Formic		Gentiobiase	Emodin monomethyl ether			Phenylethylamine
Fulvic		α-Glucosidase	Erythroglaucin			Terrein
Fumaric		β-Glucosidase	Flavoglaucin			Vitamins
Fusarinic		Histozyme	Fulvic acid			Etc.
Gallic		Inulase	Helminthosporin			Arsenic compounds:
Gentisic		Invertase	Hydroxyisohelminthosporin			Methyldiethylarsine
Glaucic		Lactase	Monascoflavin			Dimethylallylarsine
Glauconic		Lecithinase	Monascorubin			Trimethylarsine, etc.
d-Gluconic		Lipase	Ochracin			Chlorine-containing compounds:
Glucuronic		Maltase	Oösporin			Erdin
Glycolic		Melezitase	Phoenicin			Geodin
Glyoxylic		Nuclease	Physcion			Griseofulvin
2-Hydroxymethylfurane-5-carboxylic		Protease	Ravenelin			
Isovaleric		Raffinase	Rubrofusarin			
Itaconic		Rennet	Tritisporin			
Kojic		Sulphatase				
γ-Ketopentadecoic		Tannase				
Lactic		Trehalase				
Luteic		Urease				
Malic		Zymase				
d-Mannonic						
l-γ-Methyltetronic						
Methyl salicylic						
Minioluteic						
Mycophenolic						
Oxalic						
Penicillic						
Puberullic						
Pyruvic						
Spiculisporic						
Succinic						
Terrestric						

Sterigmata (singular, "sterigma") produce conidia or clusters of other sterigmata. When there are two series of sterigmata present, the first series, the one adjacent to the vesicle, is designated as the "primary

sterigmata." These give rise to and support the second series of sterigmata, which are called "secondary sterigmata." In the latter case, conidia are produced by the secondary sterigmata.

The conidium (spore) is produced by an elongation and cell division of the sterigma. A crosswall appears and the newly formed cell matures. Other conidia are produced by the same sterigma in a similar manner with the result that an unbranched chain of conidia appears, of which the outermost are the eldest. These conidia vary among different species in respect to color, size, and shape.

Spore heads vary in respect to arrangement, color, size, and shape. Heads may be globose, like *A. niger;* hemispherical, like *A. terricola* var. *americana;* elliptical; clavate, like *A. clavatus* (the head in this case is clavate or elliptical); columnar, like *A. flavipes;* or of some other characteristic shape.

Perithecia are produced by only a few species. The perithecium[1] is a thin-walled receptacle, or fruit body, commonly globose or flask-shaped, closed at maturity, which produces ascospores. The ascospores are liberated when the thin walls of the perithecium break.

Sclerotia, which are hard masses formed from the mycelium, usually possess somewhat characteristic markings and colorations. They are produced by some species of *Aspergillus*.

Some Important Aspergilli.—Members of the *A. flavus-oryzae* group, especially strains of the *A. oryzae* series, have large industrial importance, particularly in the Orient. In Japan, *A. oryzae* is used to saccharify rice starch in the manufacture of sake and other alcoholic liquors; in the manufacture of *shoyu* (soy sauce); in the manufacture of *miso*, a soybean product used as a breakfast food; and in the preparation of *mizaume*, a sugar sirup made from rice. *A. oryzae* is used also in the preparation of enzyme mixtures, which appear on the market under such trade names as Takadiastase, Polyzyme, Digestin, Oryzyme, and Kashiwagidiastase. Kojic acid is produced by the same mold.

Strains of *A. niger* are used in three industrial fermentations (refer to Chaps. XXV, XXVI, and XXVIII). This mold may cause serious damage in the textile industry (see Chap. XXXV).

A. tamarii is used in the Orient in the production of *tamari* sauce from soybeans or from soybeans mixed with rice.

Divisions of the Penicillia.—The penicillia are divided into four main groups by Thom:[2] the *Monoverticillata*, the *Biverticillata-symmetrica*, the *Polyverticillata-symmetrica*, and the *Asymmetrica*. These groups are still further divided in some cases. The basis for the principal divisions is the type of branching in the penicillus or spore head. Colony characteristics furnish a basis for further subdivision.

[1] Smith, *op. cit.*

[2] Thom, C., "The Penicillia," The Williams & Wilkins Company, Baltimore, 1930.

Figure 37 shows a division of the penicillia proper into main groups. Some important species are listed.

The *Monoverticillata* contain one cluster, whorl, or verticil of sterigmata (the conidia-producing organs of the mold) supported by the conidiophore. In this group belong the *Citromyces* of Wehmer, molds that have

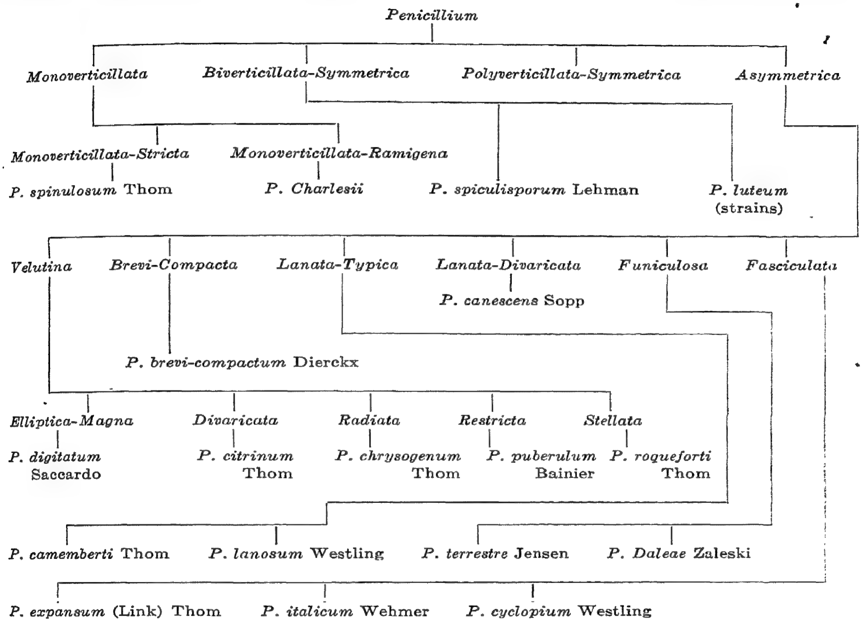


FIG. 37.—A subdivision of the genus *Penicillium*.

the ability to produce citric acid; and *P. Charlesii* and *P. spinulosum*, molds studied for their biochemical characteristics by Raistrick and his associates.

In the *Biverticillata-symmetrica*, a verticil or whorl of sterigmata is supported by short branches or metulae (singular, metula), which are arranged symmetrically about the axis of the conidiophore in the form of a verticil or whorl. *P. luteum-purpurogenum*, a mold that produces gluconic acid, and *P. pinophilum*, a mold that stains wood,[1] are members of this group.

There are three or more stages of branching in the *Polyverticillata-symmetrica* group, which contains only a few unimportant molds.

In the *Asymmetrica*, the spore head, or penicillus, is branched asymmetrically about the axis of the conidiophore. This group is the largest

[1] *op. cit.*

of the four and includes species of economic importance: molds producing characteristic changes in cheeses; molds causing destruction of fruits; and molds with the ability to produce gluconic acid or other compounds from nutrient glucose solutions.

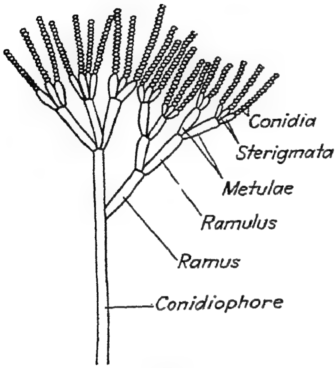


FIG. 38.—Diagram of the penicillus of a *Penicillium*. (*Adapted from Thom, "The Penicillia," The Williams & Wilkins Company, Baltimore*, 1930.)

Two species of *Penicillium, P. italicum* and *P. digitatum*, cause much damage to citrus fruits. Invasion of the fruit occurs through wounds. Careful handling and packing, the use of special protective treatments[1] to prevent infection of the fruit, and the use of wrapping material treated with diphenyl[2] do much to prevent losses due to these molds.

P. italicum produces a soft rot of citrus fruits. The colonies on the fruit are blue-green in color owing to the spores.

P. digitatum (*P. olivaceum*) causes the infected fruit to shrivel and dry up.[3] Conidia of the mold are dull yellow-green or olive-green in color.

Soft rot of stored apples and pears is caused by *P. expansum*. Coremia formation is characteristic of the mold. The coremia, *i.e.*, bundles of conidiophores, are green to gray-green in appearance.

The Genus Rhizopus.—Species of *Rhizopus* are of industrial value to man. *Rhizopus oryzae* and other species have the ability to produce *d*-lactic acid from nutrient sugar media (see Chap. XXVII) while *R. japonicus, R. delemar*, and other species have been used in the Amylo process for converting starches to sugars (see page 48).

On the other hand, certain species of *Rhizopus* are the cause of the destruction of plant products. *R. nigricans* is the cause of soft rot in sweet potatoes, especially during storage. The same mold also causes rot in fruits, for example, the soft watery rot of strawberries, which is known also as "leak." *R. necans* produces a bulb rot in the lily, *R. nodosus* a boll rot of cotton.[4]

General Characteristics of the Genus.—Reference to the accompanying figure will aid in understanding the general description of the genus

t, S. C., and B. E. PROCTOR, "Food Technology," McGraw-Hill Book Company, Inc., New York, 1937.

[2] *Ind. Eng. Chem.* (*News Ed.*), **17**: 210 (1939).

[3] SMITH, *op. cit.*

[4] HEALD, F. D., "Introduction to Plant Pathology," McGraw-Hill Book Company, Inc., New York, 1937.

Rhizopus. Erect aerial sporangiophores arise from the nodes, the places where the stolons or runners are attached to the substrate or surroundings

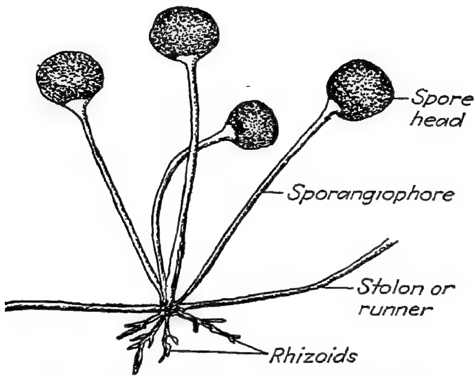


Fig. 39.—Diagram of *Rhizopus nigricans*.

by means of rhizoids. Spore heads are borne at the upper ends of the fruiting hyphae (sporangiophores) in clusters or whorls. Groups of sporangiophores are connected by hyphae of a vegetative nature, the

Table 69.—Some Enzymes Formed by Some Industrially Important Molds

Aspergillus niger	*Aspergillus oryzae*	*Penicillium camemberti*
Amylase (diastase)	Amidase	Amidase
Cellobiase	Amylase	Amylase
Emulsin	Catalase	Emulsin
Gentianase	Cytase	Erepsin
Gentiobiase	Dextrinase	Inulase
Inulase	Emulsin	Invertase
Invertase	α-Glucosidase	Lactase
Lipase	β-Glucosidase	Lipase
Maltase	Histozyme	Maltase
Melezitase	Inulase	Nuclease
Nuclease	Invertase	Protease
Protease	Lactase	Raffinase
Raffinase	Lecithinase	
Rennet	Lipase	
Tannase	Maltase	
Trehalase	Protease	
Zymase	Rennet	
	Sulphatase	
	Tannase	

stolons, which may be regarded as distributing hyphae. The rhizoids are vegetative hyphae, which function as anchors.

Thick-walled spores (chlamydospores) are formed by some species, for example, *R. oryzae*.

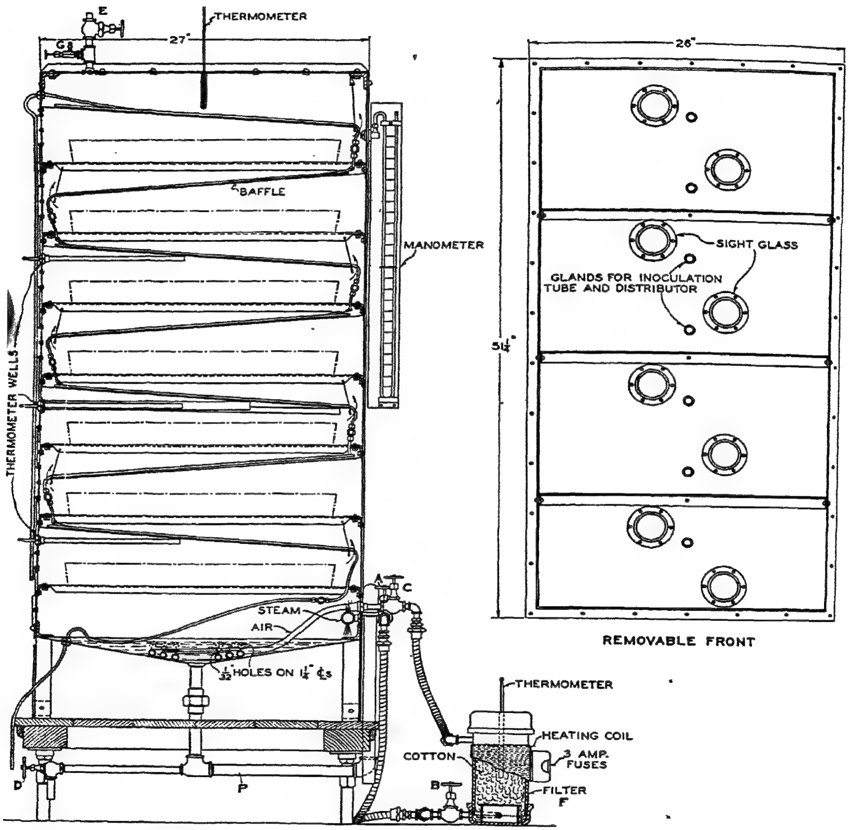


FIG. 40.—Cabinet incubator. [*From Ward, Lockwood, May, and Herrick: Ind. Eng. Chem.*, **27**: 318 (1935.)]

Mold Enzymes.—Various kinds of enzymes are produced by different species of molds. A list of some of the enzymes produced by strains of the *Aspergillus niger* and *A. oryzae* groups and *Penicillium camemberti* is given in Table 69.

With certain exceptions, molds have not been thoroughly studied in respect to the kinds of enzymes elaborated and the conditions under which they are produced. This field of research offers many possibilities.

The interested reader will find considerable information dealing with the enzymes of molds in the text prepared by Waksman and Davison.[1]

Apparatus for Cultivating Molds.—From time to time special equipment has been devised for cultivating molds or for carrying out mold fermentations on a large scale in the laboratory. Birkinshaw and his associates have described an incubator for the large-scale growth of molds.[2] An apparatus for the large-scale laboratory cultivation of molds has been described by Peterson and his coworkers.[3] A sterilizer-incubator, somewhat similar to the ones mentioned above, was described by Ward and his collaborators[4] (see Fig. 40).

In the chapter on the gluconic acid fermentation, other types of equipment used in fermentation are described or referred to.

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[1] Waksman, S. A., and W. C. Davison, "Enzymes," The Williams & Wilkins Company, Baltimore, 1926.

[2] Birkinshaw, J. H., J. H. V. Charles, C. H. Lilly, and H. Raistrick, *Trans. Roy. Soc. (London)*, **B220**: 136–138, 366–367 (1931).

[3] Peterson, W. H., L. M. Pruess, H. J. Gorcia, and H. C. Greene, *Ind. Eng. Chem.*, **25**: 213 (1933).

[4] Ward, G. E., L. B. Lockwood, O. E. May, H. T. Herrick, *Ind. Eng. Chem.*, **27**: 318 (1935).

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CHAPTER XXV

THE CITRIC ACID FERMENTATION

The production of citric acid by fermentation on a commercial basis has been a highly important achievement in the field of industrial microbiology. It has made the United States self-sufficient in respect to the citric acid supply and greatly changed the commerce of the world in citric acid and calcium citrate.

Citric acid ($COOH \cdot CH_2 \cdot C(OH) \cdot COOH \cdot CH_2 \cdot COOH$) was first isolated from lemon juice and crystallized as a solid by Scheele in 1784. It is found as a natural constituent of citrus fruits, pineapples, pears, peaches, figs, and other fruits and tissues. The citric acid extracted from these products is known as natural citric acid in contrast to fermentation citric acid. Cull lemons, limes, and pineapples are the principal sources of natural citric acid, which is produced chiefly in Italy, especially Sicily, and also in California, Hawaii, and the West Indies.

In 1922, Italy produced approximately 90 per cent of the world's supply of calcium citrate, which is used in citric acid manufacture. Much of this calcium citrate was exported to the United States, England, and France. Since 1927, however, very little calcium citrate or citric acid has been imported into this country. Several factors have been responsible for this change: the production of citric acid by mold fermentation, an increase in the numbers of trees bearing lemons in the United States, importations of concentrated lemon juice, and high import duties.

Wehmer, in 1893, first described citric acid as a product of mold fermentation. Two molds, which he designated as *Citromyces pfefferianus* and *Citromyces glaber* (classified by Dr. Thom as penicillia), produced the acid from nutrient sucrose solutions containing calcium carbonate. Later Wehmer reported the formation of citric acid by *Penicillium luteum* and *Mucor piriformis,* but it is interesting to note that he believed that the black aspergilli produced only oxalic acid. This idea was disproved by Thom and Currie. Nevertheless, oxalic acid is generally an accompaniment of the citric acid produced.

In 1917, Currie, of the U.S. Department of Agriculture, published the results of a fundamental research concerning the production of citric acid by a strain of *Aspergillus niger.* Doelger and Prescott, in 1934, corroborated the results of Currie and made other valuable contributions concerning the fermentation.

The literature of the past two decades contains many references to the citric acid fermentation, but no attempt will be made to review it in detail. Certain selected references giving the principal facts gained by research will be found at the end of this chapter. The interested student is urged to consult some of the papers cited.

Significant Factors in Fermentation.—The organism, the correct interrelation of the various constituents of the medium—sugar and inorganic salts—the pH, the ratio of surface area to volume of solution fermented, the oxygen supply, and the temperature have much to do with the nature and magnitude of the yield of end products recovered from a fermentation medium. By adjusting the salts and pH carefully, it is possible to produce citric acid with a negligible or small amount of oxalic acid.

Organisms.—Since the historic researches of Wehmer, it has been shown that a large number of fungi have the ability to produce citric acid. Some of the fungi produce small yields; some produce undesirable substances; some, on account of their unstable cultural characteristics, would be unsatisfactory for use on a commercial basis. Thus the choice of a strain is of great importance.

Aspergillus niger, *A. clavatus*, *Penicillium luteum*, *P. citrinum*, *Paecilomyces divaricatum*, *Mucor piriformis*, *Ustulina vulgaris*, and another species of *Mucor* have been used to produce citric acid in the laboratory or on a commercial scale.

Strains of the *Aspergillus niger* group of molds have usually given most successful results, both in the laboratory and on an industrial basis. Many of these molds produce high yields, possess fairly uniform biochemical characteristics, are easily cultivated, and produce a negligible quantity of undesirable end products.

Sugars.—Many organic substances, among them 2-, 3-, 4-, 5-, 6-, 7-, and 12-carbon compounds (principally sugars), may be fermented to citric acid. Maximum yields have been secured, usually, from sucrose and fructose. Occasionally glucose, under certain circumstances, has given high yields, comparable with those from sucrose. For industrial fermentations, sucrose and technical glucose are best; maltose and molasses are less desirable.

In general, a high concentration of sugar is required to produce high yields of citric acid. Solutions with concentrations of 14 to 20 per cent may be used. Currie advocated the use of 125 to 150 g. of sucrose per liter. Doelger and Prescott obtained highest yields when using a concentration of 140 g. of sucrose per liter in fermentations that were allowed to run for 9 to 12 days. They found that if more than 15 per cent of sugar was used, a greater amount of sugar than normal (less than 3 per cent) remained unconverted to citric acid. Substitution of part of the sucr

by fructose or glucose, such that the concentrations of these sugars represented 1 to 5 per cent (out of the total of 14 per cent) resulted in lower yields of citric acid than were obtained from controls containing sucrose alone. Partial hydrolysis of sucrose during sterilization likewise resulted in lower yields.

Inorganic Salts.—In addition to the carbon, hydrogen, and oxygen supplied by the carbohydrate, also nitrogen, potassium, phosphorus, sul-

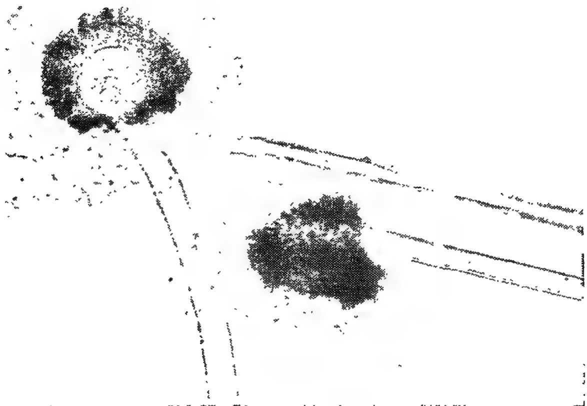


FIG. 41.—Typical spore-bearing heads of *Aspergillus niger*. Club-shaped head bears two rows of columnar cells with spores forming at the ends of the second row of cells. [*From W. P. Doelger and S. C. Prescott, Ind. Eng. Chem.*, **26**: 1142 (1934).]

phur, and magnesium are indispensable in the fermentation medium, according to Currie and Doelger and Prescott. Currie[1] found that the most favorable medium for producing citric acid contained the following:

	Grams per Liter
Sucrose	125 –150
NH_4NO_3	2 – 2.5
KH_2PO_4	0.75– 1.0
$MgSO_4 \cdot 7H_2O$	0.20– 0.25
HCl to pH 3.4–3.5 (5–4 cc. N/5 HCl)	

Doelger and Prescott[2] found the following medium to be most satisfactory, for the strain of *Aspergillus niger* used produced high yields of citric acid with less than 2 per cent of oxalic acid from this medium:

[1] CURRIE, J. N., *Jour. Biol. Chem.*, **31**: 15–37 (1917).

[2] DOELGER, W. P., and S. C. PRESCOTT, *Ind. Eng. Chem.*, **26**: 1142 (1934).

	Grams per Liter
Sucrose	140
NH_4NO_3	2.23
K_2HPO_4	1.00
$MgSO_4 \cdot 7H_2O$	0.23

(Salts and sugars are dissolved and made up to 1 liter with distilled water, adjusted to pH 2.20 to 1.60 with N/1 HCl, and sterilized at 8 to 10 lb. steam pressure per square inch for 30 min.)

If more than 2.50 g. of ammonium nitrate, 1.50 g. of potassium monohydrogen phosphate and 0.30 g. of magnesium sulphate were used, oxalic acid formation increased and the yield of citric acid was decreased. Ammonium nitrate in a concentration of more than 2.50 g. per liter caused the formation of a heavy mat. More than 0.30 g. of magnesium sulphate per liter favored sporulation. In general, high yields of citric acid were obtained when the mats were thin and the sporulation light or nearly absent; these results were secured when a minimum quantity of inorganic salts were used. Restriction of the nitrogen supply tended to cause increased yields of citric acid.

Wells and Herrick[1] report the following limits for the amounts of salts generally used in the fermentation: 0.03 to 0.1 per cent of KH_2PO_4, 0.01 to 0.05 per cent of $MgSO_4 \cdot 7H_2O$, and 0.16 to 0.32 per cent of NH_4NO_3.

In certain cases, other nitrogen-containing salts have been found to be superior to ammonium nitrate. Sodium nitrate in a 0.4 per cent concentration was found to be better than ammonium nitrate or ammonium sulphate by Porges, while Butkewitsch and Gaewskaya used potassium nitrate in a 0.35 per cent concentration in order to obtain high yields of citric acid.

The use of salts other than those mentioned above does not usually appear to be necessary, although many apparently conflicting statements concerning the value of the use of certain salts in the medium for producing high yields of citric acid appear in the literature. For example, some investigators state that iron and zinc accelerate the formation of citric acid; others are of the opinion that salts of iron and zinc stimulate growth of the mycelium without increasing the yields. Still others have shown that zinc salts have a definite inhibitory effect on citric acid production. Thus, in a few instances, it seems that iron, and possibly zinc salts, have favored acid production.

Undoubtedly, the strain of fungus used by a worker has a very important bearing on the salt requirement. That this is true has been shown by several men. Osnizkaya, using one strain of *Aspergillus niger*, obtained a marked increase in the yield of citric acid from sucrose by

[1] Wells, P. A., and H. T. Herrick, *Ind. Eng. Chem.*, **30**: 255 (1938).

the addition of 0.3 per cent magnesium nitrate, but, when using a second strain, the addition of magnesium nitrate actually caused a slight diminution in the yield. According to Quilico and Di Capua, the effect of iron on citric acid production depends on the strain of *A. niger* used. In one case the yield of citric acid was increased and in another case decreased by the addition of increasing quantities of iron.

The presence or absence of minute traces of elements in a medium may have a marked effect on the result obtained. Improved methods of analysis—the use of the spectroscope, and other precision measurements—have aided the microbiologist and chemist greatly in recent years in detecting the presence of unsuspected elements in supposedly pure compounds. An increasing amount of evidence stresses the importance of the presence of mere traces of substances, in quantities of a fraction of a part per million, for example.

pH.—The maintenance of a favorable pH is most important for the successful progress and termination of a fermentation. Currie demonstrated that by controlling the pH and the inorganic salts, the proportions of citric and oxalic acids could be varied considerably. In fact, when using conditions that favored the highest yields of citric acid, the formation of oxalic acid was nearly completely suppressed.

The use of a low pH is advantageous in that high yields of citric acid are favored, oxalic acid formation is suppressed, and the danger of contamination is minimized. Sterilization is more readily effected at a low pH. In the laboratory, successful fermentations have been carried out without resorting to heat sterilization of the medium when the initial pH was low (2.20 or below). In general, the best citric-acid-producing molds possess the greatest tolerance to low pH values.

Hydrochloric acid was used by Currie to adjust the pH of his medium to 3.4 to 3.5. Doelger and Prescott advocated the use of this acid also, for the element chlorine was found to be of distinct value as a constituent of the medium. The pH range of 1.60 to 2.20 was found to be the most satisfactory by these men; a pH value in the upper part of this range was used when distilled water was employed in the medium rather than Cambridge tap water. This indicates that for commercial production the character of the water supply should be carefully observed. From the point of view of yields of citric acid, sulphuric, nitric, and acetic acids were found to be inferior to hydrochloric acid. A quantity of formic acid sufficient to lower the pH to 3.0 prevented the mold spores from germinating.

It is not generally considered necessary to add calcium carbonate to neutralize the acids formed during fermentation. Its use favors contamination, while its absence favors higher yields and a shorter fermentation period. However, calcium carbonate has been used by

Wehmer, Chrzaszcz, and Peyros to increase the yield of citric acid. Here again the strain of *Aspergillus niger* used and its tolerance to acid may be of large significance.

Ratio of Surface Area to Volume.—In the citric acid fermentation, the conversion of sugar to citric acid is brought about by intracellular enzymes and therefore takes place within the living cells that make up the mycelial mat. Sugar passes by osmosis into the cells, while the acid diffuses out through the cells. The rate at which enzymic and diffusion processes proceed will determine the length of the fermentation period. Obviously, in a deep vessel containing a large volume, the progress of acid formation will be relatively slow, for the surface area of the mat will be small in comparison with the volume. By using shallow pans, a large surface area of mycelium is exposed to a relatively shallow layer of medium. Conversion of sugar to citric acid proceeds much more rapidly. The ratio of surface area to volume at which a maximum quantity of citric acid will be produced during the shortest fermentation period with a minimum of sugar unconverted to citric acid should be used.

The effect of varying the ratio of the volume to the surface area of the medium is illustrated in the following table from Doelger and Prescott's researches. Shallow pans of aluminum (25 by 33 cm.), of a purity of 99.80 per cent or greater, were used during the experiment. Standard medium at a pH of 2.40 was inoculated and maintained at a temperature of 26°C. until the ninth day, when analyses were carried out.

TABLE 70.—THE EFFECT OF VARYING THE RATIO OF VOLUME TO SURFACE AREA ON THE YIELDS OF CITRIC ACID[1]

Volume: surface area ratio, cc. per sq. cm.	Original volume, cc.	Sucrose per pan, grams	Final volume, cc.	Citric acid per 100 cc., grams	Total citric acid per pan, grams	Yield of citric acid to sugar, per cent
2.45	2,000	280	1,810	6.35	114.9	41.0
2.20	1,800	252	1,620	7.05	114.2	45.3
2.08	1,700	238	1,490	7.40	110.3	46.3
1.83	1,500	210	1,310	7.75	110.2	48.3
1.22	1,000	140	780	8.80	68.6	49.0

[1] DOELGER, W. P., and S. C. PRESCOTT, *Ind. Eng. Chem.*, **26**: 1142 (1934).

Agitation of the medium by a gentle or moderate shaking motion retards the rate of citric acid production, according to Doelger and Prescott. (Compare with the sorbose and dihydroxyacetone fermentations when the shallow-pan method is used.)

Oxygen Supply.—Large amounts of air have an adverse effect on the yield of citric acid, according to Porges, Doelger and Prescott, and Gudlet. The flow of small amounts of air over the mat has no injurious effect,

however, as Wells and his associates have passed sterile, humidified, carbon-dioxide-free air over mats in 2,000 cc. Erlenmeyer flasks at a rate of 15 cc. per min. and obtained results similar to those secured with controls receiving no special air supply. Too low an air supply, on the other hand, also reacts unfavorably on the yield of citric acid. Regulation of supply of air must therefore be determined experimentally for each installation of apparatus.

Temperature.—The temperature used will depend in part on the organism and the fermentation conditions. Temperatures of 25 to 35°C. are usually employed. Doelger and Prescott advocate 26 to 28°C. as the optimum temperature range. They state that the "amount of citric acid produced will be on a rising scale as the temperatures are increased from 8 to 28°C." and that at 30°C. or above "citric acid production will decrease and a greater proportion of the titrable acidity will be due to the formation of oxalic acid." The following table shows the effect of the temperature of incubation on titrable acidity and the quantity of citric acid formed. Standard medium was used in the experiment.

In 250-cc. flasks were placed 75-cc. portions, and all were inoculated from the same 10-day-old culture of mold spores (*Aspergillus niger*).

After a 10-day period of incubation, samples from each flask (10 at each temperature) were analyzed:

TABLE 71.—EFFECT OF TEMPERATURE OF INCUBATION ON TITRABLE ACIDITY AND CITRIC ACID PRODUCED[1]

Temp. of incubation, °C.	Titrable acidity, normality	Citric acid produced		Evaporation of medium, per cent
		Per flask fermentation, grams	Per 100 g. sugar, grams	
20–22	1.0024	3.37	32	24
24	1.0535	3.55	34	27
26	1.1187	3.96	38	26
28	1.1564	3.88	37	30
30–33	1.1045	2.87	27	36

[1] DOELGER, W. P. and S. C. PRESCOTT, *Ind. Eng. Chem.*, **26**: 1142 (1934).

Duration of Fermentation Period.—In the production of citric acid by the shallow-pan method, the fermentation is usually complete in 7 to 10 days.

Yields.—On the basis of the sugar consumed, a maximum yield of 90.7 per cent of citric acid was obtained from glucose by Wells, Moyer, and May. Carbon balances were prepared to show exactly how the carbon was used during the fermentations. Clutterbuck and his associates have secured yields as high as 87 per cent on a semicommercial scale.

Yields amounting to about 100 per cent on the basis of the sugar consumed have been reported by Butkewitsch and Gaewskaya. Usually, however, about 60 per cent of the weight of the sugar used in the medium may be recovered as citric acid.

Recovery of Citric Acid.—At the completion of the fermentation, the solution is drained off and the mat is pressed to remove any acid contained in it. Calcium citrate is then precipitated from a hot neutral solution. By treating the precipitate with an equivalent of sulphuric acid, the citric acid is liberated and is recovered by separating it from the calcium sulphate.

The unconverted sugar may be fermented by yeasts and the citric acid crystallized directly, in an alternate method.

Cultural Methods.—The successive transfer of spores from one lot of a medium to another of the same uniform composition may stimulate the mold to produce large yields of citric acid. Doelger and Prescott[1] have demonstrated the effect of 18 successive transfers on titrable acidity. Twelve 250-cc. flasks, each containing 75 cc. of the standard medium (14 per cent sucrose) adjusted to a pH of 2.00, were inoculated with the spores of a strain of *Aspergillus niger* from a single culture and incubated at a temperature of 26°C. for 10 days. Twelve new flasks were inoculated

TABLE 72.—AVERAGE TITRABLE ACIDITY PRODUCED WITH EIGHTEEN SUCCESSIVE SPORE TRANSFERS

No. of inoculation series	Average titrable acidity, normality	Increase in titrable acidity from low point, normality	Average spores on mat, per cent
1	0.4544	......	10.1
2	0.3438	......	53.2
3	0.2844	0.0000	32.7
4	0.3298	0.0454	14.5
5	0.3430	0.0586	9.5
6	0.3780	0.0936	8.0
7	0.3250	0.0406	6.7
8	0.4703	0.1859	5.0
9	0.4308	0.1464	5.5
10	0.4801	0.1956	7.1
11	0.3595	0.0751	32.2
12	0.5507	0.2663	3.3
13	0.5213	0.2369	3.1
14	0.5718	0.2774	3.3
15	0.4717	0.1873	1.4
16	0.6959	0.4115	1.6
17	0.6232	0.3388	3.3
18	0.8116	0.5272	1.3

[1] DOELGER and PRESCOTT, *loc. cit.*

from the spores that appeared on the mycelial mats of the first set of flasks. This process was repeated at intervals of 10 days for about 8 months. Results of this experiment are shown in Table 72.

The figures contained in the preceding table indicate a general increase in the level of titrable acidity with successive transfers. The drop in titrable acidity after the first three transfers may be ascribed to the fact that the mold had been grown previously in a 10 per cent sucrose solution and required time to become adjusted to the new concentration. After producing a titrable acidity approximately equivalent to a 1.2 N solution, there is usually no further increase in the titrable acidity.

The right-hand column of the table shows the relation of sporulation to yields. The term "average spores on mat" refers to the approximate percentage of the surface covered by spores. Those fermentations in which high yields of citric acid were obtained showed only a few spores or even a complete absence of spores. Thus sporulation may be used as a means of judging the efficiency of a fermentation.

By seeding only one-fourth to one-half of the surface area of the medium with spores, Doelger and Prescott obtained the best results. Uniform sprouting of the spores is prevented if the entire surface is seeded, with the result that unsprouted spores become embedded in the mycelial mat. It is believed that the unsprouted spores may exert a toxic effect toward citric acid production.

For a detailed discussion of the technique of the fermentation, the reader is referred to the publications of Currie, Doelger and Prescott, and others (see the bibliography at the end of the chapter).

Characteristics of the Fermentation.—Sterile nutrient sugar solutions in shallow pans are inoculated with mold spores and incubated at the most favorable temperature for fermentation. The spores commence to sprout after a few hours and within 2 to 5 days the surface of the medium is covered by a firm, mycelial mat. With the formation of the mycelial felt, citric acid production proceeds at a rapid rate, and the fermentation is usually completed in 7 to 10 days.

Figure 42[1] shows curves for titrable acidity, pH, and weight of the mat for mashes that were allowed to incubate for 20 days at 24°C.

When about 90 per cent of the sucrose originally present in the medium has been converted to other products, the rate of increase of titrable acidity diminishes. Likewise when the concentration of citric acid is greater than approximately 7 per cent, it retards the rate of increase of titrable acidity. In a normal fermentation, the titrable acidity increases up to the ninth or tenth day, at which time there will be 7 to 8 per cent of citric acid and less than 1 per cent of oxalic acid. (An 8 per cent citric acid concentration is equivalent to a 1.2 N solution.)

Citric acid is broken down unless it is recovered within a reasonable time after being produced.

Citric Acid by the Cahn Method.—A rather unusual method of citric acid production was advocated by Cahn[1] in 1934. Solid material, such as cane or beet pulp, is impregnated with sugar solutions—sucrose or molasses. The unsterilized mass is inoculated with a mold culture, the age of which is said to be relatively unimportant, and fermentation proceeds rapidly over the relatively large surface at a temperature of 20 to

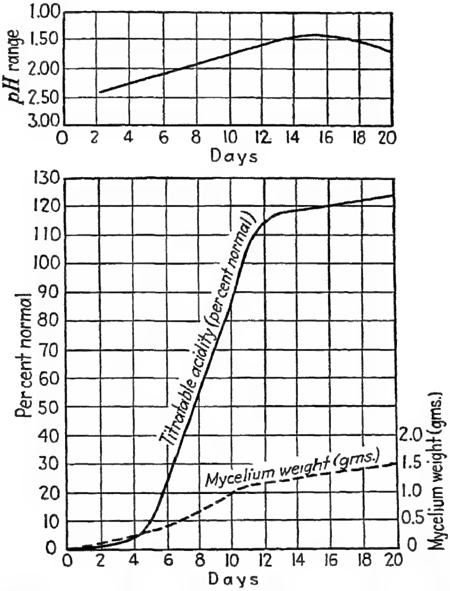


FIG. 42.—Rate of increase in titrable acidity, weight of mat, and pH range in flask fermentations. [*From W. P. Doelger and S. C. Prescott, Ind. Eng. Chem.*, **26**: 1142 (1934).]

35°C. The fermentation is usually complete in 4 days or less, at which time a yield of 55 per cent citric acid on the basis of the sucrose originally present, or 45 per cent acid, calculated on the basis of the sugar originally present in the molasses, may be expected. One pound of citric acid may be secured from 5.7 lb. of molasses and 2.75 lb. of beet pulp (beet pulp may be used only once, while cane pulp may be used more than once).

Production of Citric Acid by a Mucor.[2]—Solutions of molasses and gur have been fermented by a species of *Mucor* with the production of

[1] CAHN, F. J., *Ind. Eng. Chem.*, **27**: 201 (1935).

[2] DAS GUPTA, G. C., SAHA, K. C., and B. C. GUHA, Science and Culture **3** (No. 7): 397 (1938).

citric and oxalic acids. By controlling the conditions of the fermentation, citric acid may be obtained with little or no oxalic acid. A molasses mash containing 1 per cent added ammonium sulphate, adjusted to a pH of 4, and incubated at a temperature of 28°C. for a period of 7 days yielded 33 per cent of citric acid on the basis of the sugar.

The Production of Citric Acid on an Industrial Scale.—The earliest attempt to produce citric acid by fermentation on an industrial scale was made in Germany around the beginning of the twentieth century. The many difficulties encountered made it impossible to meet the competition offered by the citrus-fruit industry and the attempt was abandoned.

It is believed that some citric acid was produced by fermentation on a commercial basis in the United States in the year 1919, but it was not until the year 1923 that appreciable quantities were manufactured by the mycological process, the first factory in the world for the commercial production of citric acid by fermentation being erected in New York that year. During the year 1929,[1] about 7,000,000 lb. of citric acid were produced by mold fermentation in this country, while at present over 10,000,000 lb. are being manufactured annually by this method. The industry has expanded so rapidly that the United States has not only become independent of outside sources but, for a number of years, has exported large quantities of calcium citrate, principally to England. These exports have dropped markedly since 1935,[1] because England has developed her own fermentation process, the domestic demand for citric acid has become greater, and the International Citric Acid Agreement between England, France, Italy, Belgium, and Czechoslovakia has succeeded in regulating exports and maintaining prices.

England, Belgium, Czechoslovakia, and, probably, Russia now produce large amounts of citric acid by fermentation, following the successful establishment of the industry in the United States.

Citric acid is produced by mold fermentation on an industrial scale in the United States by the Charles Pfizer Company of Brooklyn; in Czechoslovakia by Montan und Industrialwerke (vorm. J. D. Starck) of Prague; in England by Kemball, Bishop and Company, Ltd., and John and E. Sturge, Ltd.; and in Belgium at Tirlemont. In Germany, J. A. Benckiser, Ludwigshafen-on-Rhine, and C. H. Boehringer Sohn, Nieder-Ingelheim-on-Rhine have undertaken the manufacture of citric acid by mold fermentation.

Although the details of the commercial production of citric acid have not been made public, it is believed that the acid is produced in shallow pans, using a strain of *Aspergillus niger*.

Uses.—Approximately 65 per cent[2] of the citric acid used in the United States is employed for medicinal purposes (citrates, etc.), 15 per

[1] WELLS, P. A., and H. T. HERRICK, *Ind. Eng. Chem.*, **30**: 255 (1938).
[2] *Ibid.*

cent in foods (flavoring extracts, soft drinks, etc.), 9 per cent in candies, and small quantities as an ingredient of ink, in silvering, in dyeing and calico printing, and in engraving, according to the U.S. Department of Commerce. Other uses are indicated.

Theories Concerning the Mechanism of Citric Acid Formation.—Although many theories have been advanced to explain the formation of citric acid from carbon-containing compounds, none proposed has satisfactorily explained all the known observations. Any theory proposed must account for the production of citric acid from 2-, 3-, 4-, 5-, 6-, 7-, and 12-carbon compounds if it is to be acceptable. It must account for high yields of citric acid from sugars, which in some instances have approached 100 per cent on the basis of the sugar consumed.

Maze and Perrier (1904) suggested that citric acid arose as a product of incomplete respiratory metabolism.

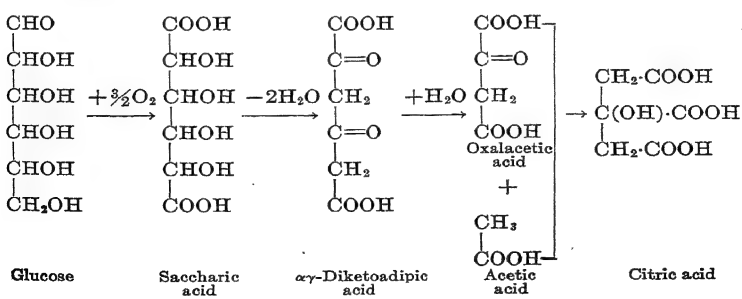
Parasaccharinic acid played an important part as an intermediate in a theory proposed by Buchner and Wustenfeld. Sugar was broken down to parasaccharinic acid, which was rearranged to form citric acid. A condensation reaction would be necessary to explain citric acid formation from some sugars (Herzog and Polotsky, 1909).

Euler (1909) and others suggested that pyruvic acid was formed from sugar and that this was decomposed to acetaldehyde and carbon dioxide. Three molecules of acetaldehyde condensed and the product was oxidized to citric acid:

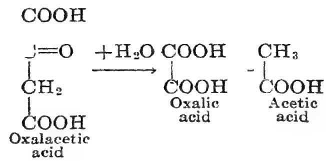
$$\underset{\text{Glucose}}{3C_6H_{12}O_6} \rightarrow \underset{\text{Pyruvic acid}}{6CH_3CO{\cdot}COOH} \rightarrow 6CO_2 + \underset{\text{Citric acid}}{2C_6H_8O_7}$$

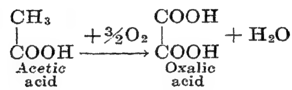
The maximum yield by this theory could only be 71.1 per cent of citric acid. Therefore this hypothesis can be eliminated on quantitative grounds alone.

In 1919, Raistrick and Clark suggested that hexose is broken down to $\alpha\gamma$-diketoadipic acid, which then becomes hydrolyzed to acetic and oxalacetic acid, these two acids combining to form citric acid.



Oxalic acid may arise from the breakdown of oxalacetic acid and the oxidation of acetic acid:



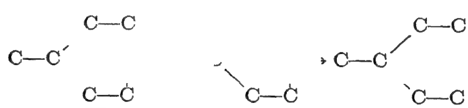


Although this theory can account for the high yields of citric acid from a hexose, it does not account for the production of this acid from substances other than a hexose, unless it is assumed that a hexose is synthesized in each case.

In 1924, Butkewitsch proposed a scheme in which glucose was directly oxidized to citric acid:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + \tfrac{3}{2}O_2 \rightarrow \underset{\text{Citric acid}}{C_6H_8O_7} + 2H_2O$$

Butkewitsch was of the opinion that glucose passed first to gluconic acid, which was subsequently transformed to glucuronic acid. The latter was then supposed to undergo an intramolecular aldol condensation to form a five-membered ring. Rupture of the ring, followed by oxidation, led to the formation of citric acid:



Wehmer (1925) suggested the possibility of the following reaction taking place, since calcium gluconate was fermented by *Aspergillus niger* to citric acid:

Glucose → Gluconic acid → Citric acid → Oxalic acid → Carbon dioxide

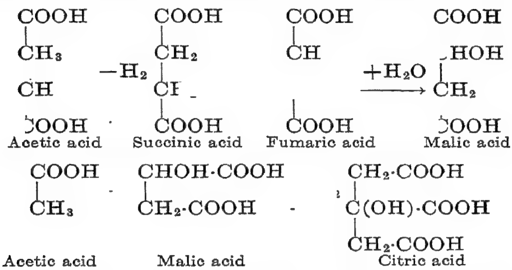
Saccharic acid was the principal intermediate product in a theory suggested by Franzen and Schmitt (1925):

$$\text{Sugar} \rightarrow \text{Saccharic acid} \rightarrow \beta\text{-}\gamma\text{-Diketoadipic acid} \xrightarrow[\text{acid rearrangement}]{\text{by benzilic}} \text{Citric acid}$$

Challenger and his associates (1927) added weight to this theory, for they isolated saccharic acid from media containing glucose that had been fermented by *A. niger*. Calcium gluconate also yielded saccharic and citric acids. Citric acid was obtained from potassium hydrogen saccha-

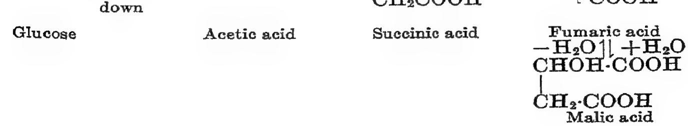
rate solutions inoculated with *A. niger*. Bernhauer, however, does not believe that saccharic acid is an intermediate product in the citric acid fermentation, as the result of his work with a large number of molds.

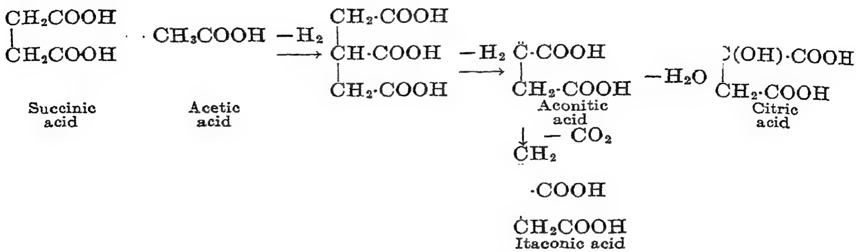
Theory of Chrząszcz and Tiukow.—From the observations (1930) that certain molds have the ability to produce citric acid, along with succinic, fumaric, and malic acids, from acetic acid and ethyl alcohol, Chrząszcz and Tiukow have formulated their scheme for the formation of these acids. Two molecules of acetic acid are dehydrogenated to 1 molecule of succinic acid, which, in turn, by loss of hydrogen, passes to fumaric acid. This acid is converted to malic acid upon the addition of 1 molecule of water. One moleucle of malic acid and 1 molecule of acetic acid combine with the loss of hydrogen to form citric acid.



It was later suggested that the initial stage in the production of citric acid from sugars was similar to that of ethyl alcohol fermentation. If this fact were true, then the maximum yield of citric acid from glucose should not exceed 71.1 per cent and the ratio of the weight of citric acid to carbon dioxide should not exceed 1.45:1. Wells and his associates have shown definitely by the use of carbon balance experiments that yields considerably higher than 71.1 per cent may be obtained from glucose, also that the ratio 1.45:1 is greatly exceeded. These general observations have been corroborated by others.

Bernhauer's Theory.—Bernhauer, Böckl, and Siebenauger (1932) have suggested that acetic acid and ethyl alcohol are formed from sugars by molds in the manner indicated by Neuberg's scheme for alcohol production by yeasts. Acetic acid is converted to citric acid through aconitic acid as follows:

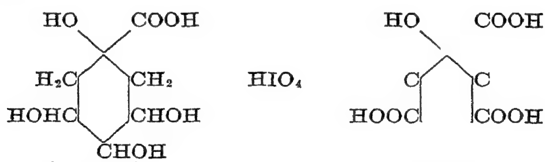




The preceding scheme is based on several facts (see the theory of Chrząszcz and Tiukow). Aconitic acid may be converted to citric acid by molds producing citric acid (one strain having formed over 20 per cent citric acid from aconitic acid) while citric acid may be transformed to aconitic acid. Methylglyoxal has been produced from sodium hexosediphosphate by aspergilli, which have the ability to form citric acid.

This theory is not acceptable, however, because the maximum yield of citric acid by this scheme cannot exceed 71.1 per cent and the ratio of the weights of citric acid and carbon dioxide cannot exceed 1.45:1. Furthermore aconitic acid has not been isolated from fermentations of sugars by molds, although *Aspergillus itaconicus* produces itaconic, citric, and gluconic acids from sucrose and fructose.

Theory of Emde.—Emde's scheme (1935) for the conversion of sucrose to citric acid through quinic acid was proposed after it had been shown by Fischer and Dangschat that quinic acid could be oxidized to citric acid by periodic acid.



Quinic acid Citric acid

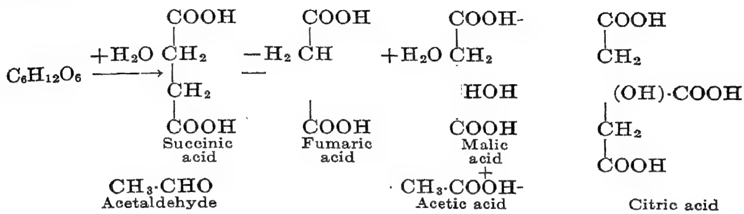
The maximum possible yield of citric acid from sucrose, according to the Emde's scheme, is 56.1 per cent:

$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}} + 5O_2 \rightarrow \underset{\text{Quinic acid}}{C_7H_{12}O_6} + 5CO_2 + 5H_2O$$

$$\underset{\text{Quinic acid}}{C_7H_{12}O_6} + \tfrac{5}{2}O_2 \rightarrow \underset{\text{Citric acid}}{C_6H_8O_7} + CO_2 + 2H_2O$$

Therefore, on the basis of yields alone, this theory is untenable. Butkewitsch, who had suggested that only molds with the ability to produce citric acid were able to ferment quinic acid, was unable to obtain citric acid from quinic acid by fermentation.

Theory of Gudlet.—The scheme proposed by Gudlet (1935) is based on the fact that glucose may split directly into a 4-carbon molecule, succinic acid, and a 2-carbon compound, acetaldehyde. (Virtanen originally suggested that this reaction might take place in the propionic acid fermentation.)



The high yields obtained from glucose, on the basis of the sugar consumed, could be explained by this scheme since decarboxylation is not concerned.

The theory that acetic acid is directly converted to succinic acid and citric acid has been attacked by Butkewitsch, Menzshinskaya, and Trofimova (1935) who hold that citric acid is formed from substances of the mycelium by *Aspergillus niger*, the process being stimulated by the addition of acetic acid. These investigators state that there is no direct connection between the consumption of acetic acid and the accumulation of citric acid, oxalic acid being the principal substance produced from the conversion of acetic acid.

By carefully weighing the mycelium, citric acid, acetic acid, and other products involved, Bernhauer and others have secured information by which they have been able to refute the hypothesis of citric acid formation from substances of the mycelium.

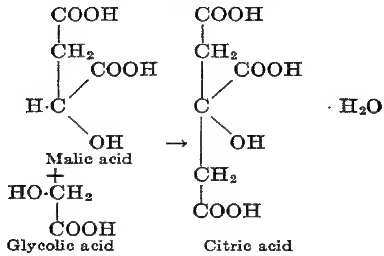
Another hypothesis concerning the fermentation of acetic acid is that it is used in the synthesis of carbohydrates which are later broken down to citric or succinic acids. This theory has little evidence for support.

When citric acid is fermented anaerobically with yeast, each molecule of citric acid yields 2 molecules of acetic acid, 2 molecules of carbon dioxide, and some formic acid. *Salmonella aertrycke* decomposes citric acid into acetic, succinic, and formic acids. These facts indicate a possible connection of citric acid with acetic, succinic, and other acids.

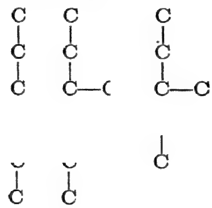
Theory of Ciusa and Brüll.[1]—Ciusa and Brüll have shown that the addition of malic acid, glycolic acid, or mixtures of the two acids to nutrient sugar solutions, adjusted to a pH of 3.5, increased the yields of citric acid produced from sugar by *A. niger*. The addition of malic acid

[1] Ciusa, R., e L. Brüll, *Ann. chim. applicata*, **29**: 3–11 (1939).

increased the yield to 332 per cent of the sugar consumed, while glycolic acid increased the yield to 132 per cent. When equimolar quantities of malic and glycolic acid were added, the yield was increased to 928 per cent. On the basis of these results, Ciusa and Brüll suggested that the last phase of the intermediary reactions in the citric acid fermentation might be a condensation of malic and glycolic acids:



The citric acid fermentation might be schematically represented as follows, according to Ciusa and Brüll:



For further details concerning the mechanism of the fermentation, consult some of the papers cited in the reference list.

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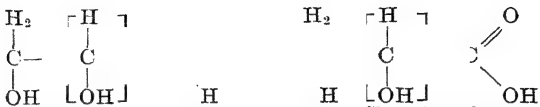
CHAPTER XXVI

THE GLUCONIC ACID FERMENTATION

Gluconic acid ($C_6H_{12}O_7$) is produced by the oxidation of the aldehyde grouping of glucose. This conversion is represented by the following equation:



Glucose Gluconic acid

Methods of Production.—By chemical means, gluconic acid may be prepared from glucose by oxidation with a hypochlorite solution. Gluconic acid may be produced, in a second method, by the electrolysis of a solution of the sugar containing a measured amount of a bromide.

Gluconic acid may be produced by the fermentation of nutrient solutions of glucose by many molds and bacteria. Great differences exist, however, between the various microorganisms in respect to their adaptability for commercial use. Some, of course, produce only small yields of gluconic acid; some may be classed as slow fermenters; some lack stable cultural characteristics.

Historical.—The production of this acid by microorganisms was first observed by Boutroux in 1878. *Mycoderma aceti* (*Acetobacter aceti*) produced a substance from glucose which was first thought to be lactic acid but which was identified two years later as gluconic acid.

Molliard, in 1922, discovered gluconic acid as a product of mold fermentation, along with citric and oxalic acids. The acid was produced in sucrose mashes as a result of the action of *Sterigmatocystis nigra* (*Aspergillus niger*). Molliard later developed some optimum conditions for the fermentation.

In 1924 Bernhauer discovered a strain of *A. niger* that would produce gluconic acid almost exclusively when in the presence of calcium carbonate. Bernhauer has shown that thin mats grown at a low temperature with a low supply of nitrogen favored the production of gluconic acid. On the other hand, higher yields of citric acid were obtained when the fermentation temperature was relatively high, the nitrogen supply high, and the mats heavy.

Preparation by Mold Fermentation.—An extensive study of the gluconic acid fermentation has been carried out over a period of years by

May, Herrick, Moyer, Hellbach, Wells, Stubbs, and others of the U.S. Department of Agriculture. In 1938 it was shown by Gastrock, Porges, Wells, and Moyer that gluconic acid could be successfully produced on a pilot-plant scale from refined corn sugar (commercial glucose). In general, gluconic acid may be produced by mold fermentation, using the shallow-pan method or using submerged mold growths under increased air pressure. The latter method is much superior to the former, not only in respect to the time required for the fermentation but also in the yield and in the ease of production.

The Shallow-pan Method.—In the shallow-pan method, a sterile nutrient glucose solution is inoculated with the mold and incubated in cabinets designed to prevent contamination. The mycelial mat develops as in the citric acid fermentation, and glucose is transformed to gluconic acid by the mold enzyme—an oxidase. During incubation the mat must not be disturbed.

Herrick and May, using *Penicillium purpurogenum* var. *rubrisclerotium* (Thom No. 2,670), found that gluconic acid could be produced to the exclusion of other acids. After considerable experimentation, they adopted the following nutrient salt solution:

	Grams per Liter of Glucose Solution
$MgSO_4 \cdot 7H_2O$	0.25
KCl	0.05
$Na_2HPO_4 \cdot 12H_2O$, or H_3PO_4	0.1
$NaNO_3$	1.0

A 20 to 25 per cent concentration of glucose was most conducive to high yields of the acid, while a temperature of 25°C. was most favorable. Lower temperatures resulted in smaller yields and required a longer time for the completion of the fermentation. At higher temperatures, the mycelial mats formed rapidly, but they sank, with resultant low yields.

The ratio of surface area to volume was important—one of 0.25 to 0.30 was found to be best for practical purposes. As the ratio of square centimeters to cubic centimeters approached unity, 82 per cent of the theoretical yield of gluconic acid was produced, but unity is an impractical ratio to employ. With a ratio of 0.16, only 30 per cent of the theoretical yield could be obtained.

Agitation of the glucose solution was considered. When the concentration of sugar was low, agitation was advantageous. The effect of agitation decreased with the increase of sugar concentration. There was no advantage to be gained from agitating a 20 to 25 per cent solution of glucose.

A pH range of 3 to 6.4 was satisfactory for the fermentation. Altering the pH values from this range brought about no advantages.

The rate of oxidation of glucose was greatest between the fifth and ninth days. When the culture liquor in a fermentation was replaced with a fresh, sterile glucose solution, an active oxidation commenced at once.

Under the foregoing conditions yields of 55 to 65 per cent of the theoretical were produced.

Moyer, May, and Herrick[1] discovered a mold (secured from the collection of Dr. Thom) which possessed biochemical and vegetative vigor, qualities lacking in the culture of *Penicillium luteum purpurogenum* investigated earlier, and which produced good yields of gluconic acid. Out of the more than 50 *Penicillium* species investigated, this mold, *P. chrysogenum*, culture 5,034.11, showed the greatest capacity for producing gluconic acid. The following medium was used for the production of gluconic acid under nearly optimum conditions: 20 to 25 per cent commercial glucose, and 3.00 g. $NaNO_3$, 0.300 g. KH_2PO_4, and 0.250 g. $MgSO_4 \cdot 7H_2O$ per liter. A surface area to volume ratio of 0.4 to 0.5 was used. Under the foregoing conditions, 60 per cent of the glucose was oxidized to gluconic acid in 8 to 10 days at 30°C. Ferric chloride acted as a stimulant to growth and acid production when nutrients of high purity were used.

Selection of Pan.—In the production of gluconic acid through fermentation by molds, using the shallow-pan method as described above, a very important consideration is that of the selection of an appropriate material for the construction of the pan. Several requirements must be satisfied, namely, noncorrosiveness to acid, lack of toxicity, low cost, and durability. Iron, zinc, and ordinary aluminum are attacked by acid. Nickel, lead, copper, and monel metal are somewhat toxic. Glass would be satisfactory but it is easily broken and expensive. Block tin and bakelite may be used, but they are also expensive. Iron, when enameled, is satisfactory, but it is easily chipped, unwieldy, and expensive. Lacquers are not good. Aluminum of a high purity, 99.45 per cent aluminum, and containing less than 0.1 per cent of copper and manganese has given good results.

Aeration, Submerged Growths.—Schreyer in 1928 demonstrated that agitation, aeration, and the use of calcium carbonate ($CaCO_3$) increased the yield of gluconic acid from four to six times that of cultures which were not aerated. *Aspergillus fumaricus* was used in glucose solutions. The yield of citric acid by this organism was not altered by the change in conditions. Thies (1930) used the same mold but bubbled oxygen instead of air through the medium and obtained similar results. Currie, Kane, and Finlay (1933) reported yields of gluconic acid as high as 90 per

[1] Moyer, A. J., O. E. May, and H. T. Herrick, *Centr. Bakt. Parasitenk.*, Abt. II, **95**: 311 (1936).

cent when the mold growth was submerged, when the medium was maintained in a high degree of agitation by means of a stirring device, and when air was drawn in large quantities into the solution.

Laboratory Scale.—In work carried out by the Color and Farm Waste Division of the U.S. Department of Agriculture,[1] gluconic acid was produced in high yields by submerged growths of *Penicillium chrysogenum* under increased air pressure. The medium used contained 3 g. of $NaNO_3$, 0.15 g. of KH_2PO_4 and 0.125 g. $MgSO_4{\cdot}7H_2O$ and the equivalent of 20 per cent of pure glucose per liter. The addition of 1 g. of calcium carbonate ($CaCO_3$) for each 4 g. of glucose caused an increase in the yields of acid and likewise served as a supporting medium.

The fermentation was carried out in 500-cc. gas-washing bottles with sintered-glass false bottoms. In each bottle, 200 cc. of nutrient medium were sterilized (at 15 lb. pressure for 15 min.), cooled, and inoculated with mold spores. Calcium carbonate, when used, was sterilized separately and added aseptically. Filtered, humidified air was permitted to enter through the sintered-glass bottoms of the bottles at a controlled rate (40 cc. per min.), thus providing agitation and aeration. Under the foregoing conditions, 80 to 87 per cent yields of gluconic acid were obtained at a temperature of 30°C. when the air pressure was maintained at 3 atmospheres or above (in an autoclave) and calcium carbonate was used in the ratio indicated.

The Rotary Drum (Laboratory Size).—In 1935, Herrick, Hellbach, and May[2] described the rotary drum, an apparatus for producing gluconic acid by submerged mold growths. The drum was developed in an attempt to find the most suitable type of equipment for the industrial production of gluconic acid by molds. It was constructed of aluminum containing less than 0.1 per cent of manganese and copper. Buckets and baffles were attached to the inside shell of the drum, which served to bring the oxygen of the air into intimate contact with the nutrient glucose solution and the mold. Facilities were available for sterilizing the drum with steam before charging it with the medium, calcium carbonate ($CaCO_3$), and inoculum.

During operation, sterilized air is introduced through an inlet and allowed to accumulate until the desired pressure is built up in the drum. Thereafter a definite quantity of air flows into and out of the drum at a constant rate. The drum is rotated by means of belts applied to its outside flanges.

The rotary drum is shown in the accompanying photograph (Fig. 43).

[1] May, O. E., H. T. Herrick, A. J. Moyer, and P. A. Wells, *Ind. Eng. Chem.*, **26**: 575 (1934).

[2] Herrick, H. T., R. Hellbach, and O. E. May, *Ind. Eng. Chem.*, **27**: 681 (1935).

Initial experimentation with the rotary drum indicated that the time required for the fermentation was considerably reduced by its use. The

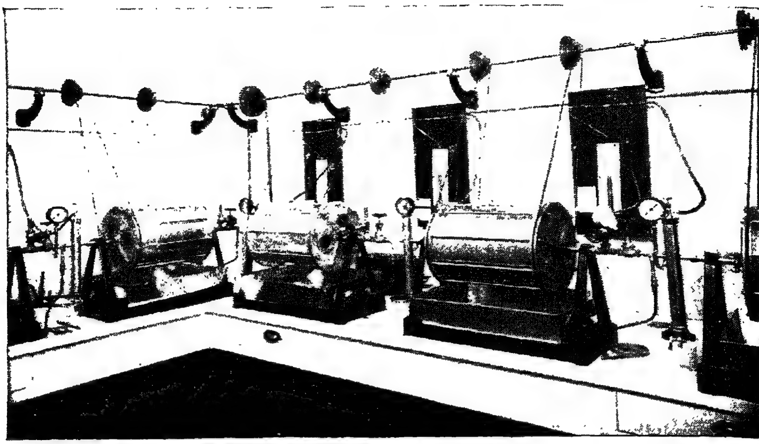


FIG. 43.—Laboratory-scale fermentation apparatus. [*Gastrock, Porges, Wells, and Moyer, Ind. Eng. Chem.*, **30**: 782 (1938).]

following table compares the production of gluconic acid by different methods.

TABLE 73.—THE PRODUCTION OF GLUCONIC ACID BY DIFFERENT METHODS[1]

Type of fermentation	Organism	Fermentation vessel	Yield of acid (theoretical),[2] per cent	Fermentation period, days
Surface..........	*Penicillium luteum purpurogenum*	Shallow pan (aluminum)	57.4	11
Submerged (pressure).	*P. chrysogenum*	Glass bottle (sintered glass, false bottom)	80.4	8
Submerged (pressure).	*P. chrysogenum*	Rotary drum (aluminum)	80.0	2.2

[1] WELLS, P. A., A. J. MOYER, J. J. STUBBS, H. T. HERRICK, and O. E. MAY, *Ind. Eng. Chem.*, **29**: 653 (1937).

[2] From 20 per cent glucose solutions.

Details of the Rotary-drum Process (Laboratory Scale).—In the rotary-drum process, the selection of an appropriate organism, the maintenance of an optimum oxygen supply, the composition of the various media employed, and the use of calcium carbonate in the process are important.

THE ORGANISM.—*Penicillium chrysogenum*, an organism used in producing gluconic acid by submerged growths under pressure, does not readily produce the large quantities of spores required for inoculating mashes. *Aspergillus niger* (strain No. 67 of the Industrial Farm Products Research Division) was selected by Wells, Moyer, and their associates because it possessed certain desirable characteristics—it readily produced spores and uniform fermentations.

THE OXYGEN SUPPLY.—The effectiveness of the oxygen supply in the rotary-drum process is controlled by three factors: agitation, air flow, and air pressure. Upon the efficiency of these factors depends, largely, the success of the fermentation, provided the medium and inoculum are satisfactory.

Optimum results were obtained when the rotation of the drum (see photograph on page 382) was 13 r.p.m. Higher speeds produced exces sive frothing of the medium. Air flows of 400 to 1,200 cc. per min. for a volume of 3,200 cc. of mash were satisfactory. (Costs increase as the amount of air used increases.) By maintaining the gauge at pressures of 30 to 45 lb. per sq. in., excellent results were obtained. A pressure of 30 lb. per sq. in. was considered most practical, however, since higher pressures increased the danger of damaging the equipment and producing leaks. The following table illustrates the effect of air pressure on the fermentation of glucose to gluconic acid by *Aspergillus niger:*

TABLE 74.—THE EFFECT OF VARIOUS AIR PRESSURES ON GLUCONIC ACID YIELDS[1]
(Air flow, 1,200 cc. per min.; speed, 13 r.p.m.; volume of medium, 3,200 cc.; glucose available, 495 g.; fermentation period, 18 hr.; temperature, 30°C.)

Gauge pressure, lb. per sq. in. (kg. per sq. cm.)	Glucose consumed, grams	Gluconic acid produced, grams	Gluconic acid yield based on	
			Glucose consumed, per cent	Glucose available, per cent
5 (0.35)	178	173	89.1	32.1
15 (1.05)	257	258	92.1	47.9
30 (2.11)	336	351	93.0	65.1
45 (3.16)	429	454	97.1	84.2

[1] WELLS, P. A., A. J. MOYER, J. J. STUBBS, H. T. HERRICK, and O. E. MAY, *Ind. Eng. Chem.*, **29**: 653 (1937).

MEDIA.—The effect of variations in the nature and quantities of the nutrient salts and other substances and in the amount of glucose on the results obtained with a given organism is well illustrated in the gluconic acid fermentation. A medium of one composition is used for maintaining the growth of the culture, a second medium for inducing sporulation, a third medium for stimulating germination, and a fourth medium for the

production of gluconic acid. Inasmuch as the media used for the production of gluconic acid on a pilot-plant scale vary but slightly from those used for the preparation of the acid on a laboratory scale,[1] a summary of the former will be given (courtesy of the authors and editors).

TABLE 75.—SUMMARY OF MEDIA USED FOR GLUCONIC ACID PRODUCTION BY *Aspergillus niger*[1]

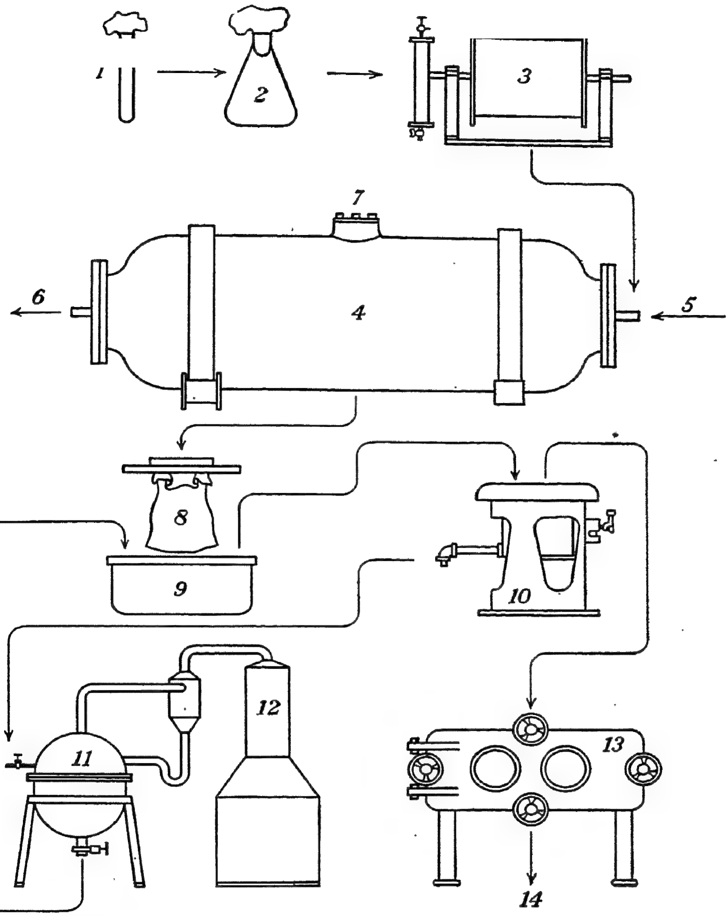
Ingredient	culture	sporulation	germination	fermentation
Grams per liter:				
Refined corn sugar[2].	30.0	50.0	100.0	Varies
$MgSO_4 \cdot 7H_2O$	0.10	0.12	0.25	0.156
KH_2PO_4.	0.12	0.144	0.30	0.188
	None	0.56	0.80	0.388
	0.225	None	None	None
Peptone	0.25	0.20	0.02	None
Potatoes	200	None	None	None
Agar	20.0	1.5	None	None
$CaCO_3$	4.0	None	37.5*	26.0*
Beer, cc. per liter	None	45	40	None
Kind of water	Distilled	Distilled	Tap	Tap

[1] GASTROCK, E. A., N. PORGES, P. A. WELLS, and A. J. MOYER, *Ind. Eng. Chem.*, **30**: 782 (1938).
[2] Refined corn sugar, containing 91.5 per cent of dextrose and corresponding closely to dextrose monohydrate, was used in almost all the research.
* Separately sterilized.

Technique of Preparing Inoculum.—*Aspergillus niger*, strain 67, is cultured for 7 days at 30°C. on slants of medium *A*. These slant cultures are used to inoculate at least twenty 1-liter Erlenmeyer flasks, each containing 150 cc. of medium *B*. The flasks are incubated at 30°C. for 7 days and then the spore-bearing mycelium is aseptically transferred to a vessel containing 17 liters of medium *C*, where it is thoroughly broken up by means of a mechanical agitator. The contents of the flask are divided into two portions, and each is placed in a small rotary drum—the total charge in each drum occupying approximately one-third of its total volume. The following conditions are maintained during germination:

Air pressure	30	lb. per sq. in. gauge (155 mm. Hg).
Air flow	375	cc. per liter per min.
Speed of rotation	5.8 r.p.m.	
Temperature	30°C.	
Time	24	hr.

[1] MOYER, A. J., P. A. WELLS, J. J. STUBBS, H. T. HERRICK, and O. E. MAY, *Ind. Eng. Chem.*, **29**: 777 (1937).



1. Tube culture.
2. Sporulation flasks.
3. Small rotating aluminum germination drums (17-liter volume).
4. Large rotating aluminum drum fermenter (540-liter volume).
5. Air inlet; water for fermentation solution and inoculum from 3 are also introduced here.
6. Air outlet.
7. Handhole; commercial dextrose, nutrients, and sterile calcium carbonate are added here.
8. Bag filter.
9. Aluminum tank for neutralization and crystallization; calcium hydroxide milk is added here.
10. Centrifuge (stainless-steel basket, aluminum-lined curb).
11. Vacuum evaporator for mother liquors.
12. Condenser.
13. Vacuum drier.
14. To calcium gluconate storage.

FIG. 44.—Flow sheet of gluconic acid production. [*Gastrock, Porges, Wells, and Moyer, Ind. Eng. Chem.*, **30**: 782 (1938).]

By permitting the spores to germinate in medium *C*, the fermentation period is shortened and less inoculum is required for a given volume of fermentation medium *D*.

The Pilot-plant Fermenter.—The solutions containing the germinated spores are transferred aseptically to a large-scale fermenter containing medium *D*. Wells, Lynch, Herrick, and May[1] have described this fermenter. The drum is 3 by 6 ft. and is constructed of aluminum sheet, containing 99.5 per cent aluminum and less than 0.1 per cent of copper, iron, and manganese. End castings are made of an aluminum-silicon

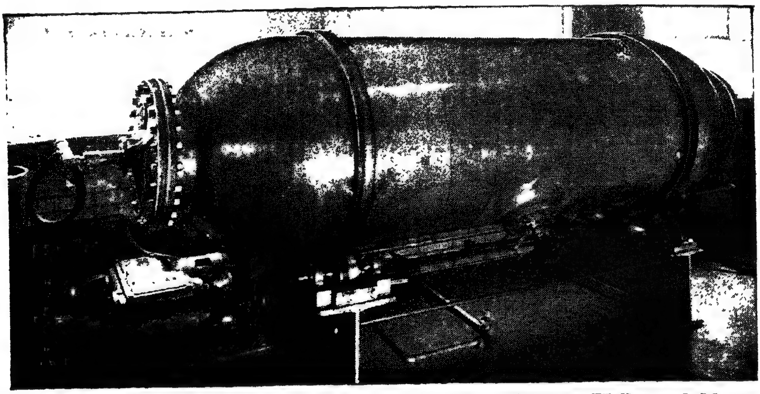


FIG. 45.—Large-scale fermentation apparatus. [*Gastrock, Porges, Wells, and Moyer, Ind. Eng. Chem.*, **30**: 782 (1938).]

alloy. The tank holds 420 gal., but ordinarily about 140 gal. of charge are added—larger charges increase the fermentation time. Buckets and baffles are welded to the interior of the shell and function as in the small rotary fermenters. Facilities are provided for sterilizing the drum and its contents. An aluminum pipe, which is attached to the drum's interior surface and looped back and forth around the ends of the buckets and baffles, extends around one-third of the periphery of the drum on the portion opposite the hand hole. By connecting one end of the tubing to a steam line and the other to a drain, steam may be supplied for sterilization purposes (Fig. 46). Means are available for filling and emptying the drum. Sterile, humidified air is passed through the drum under pressure. For further details, the reader is referred to the papers cited in the foregoing paragraphs.

[1] WELLS, P. A., D. F. J. LYNCH, H. T. HERRICK, and O. E. MAY, *Chem. & Met. Eng.*, **44**: 188 (1937).

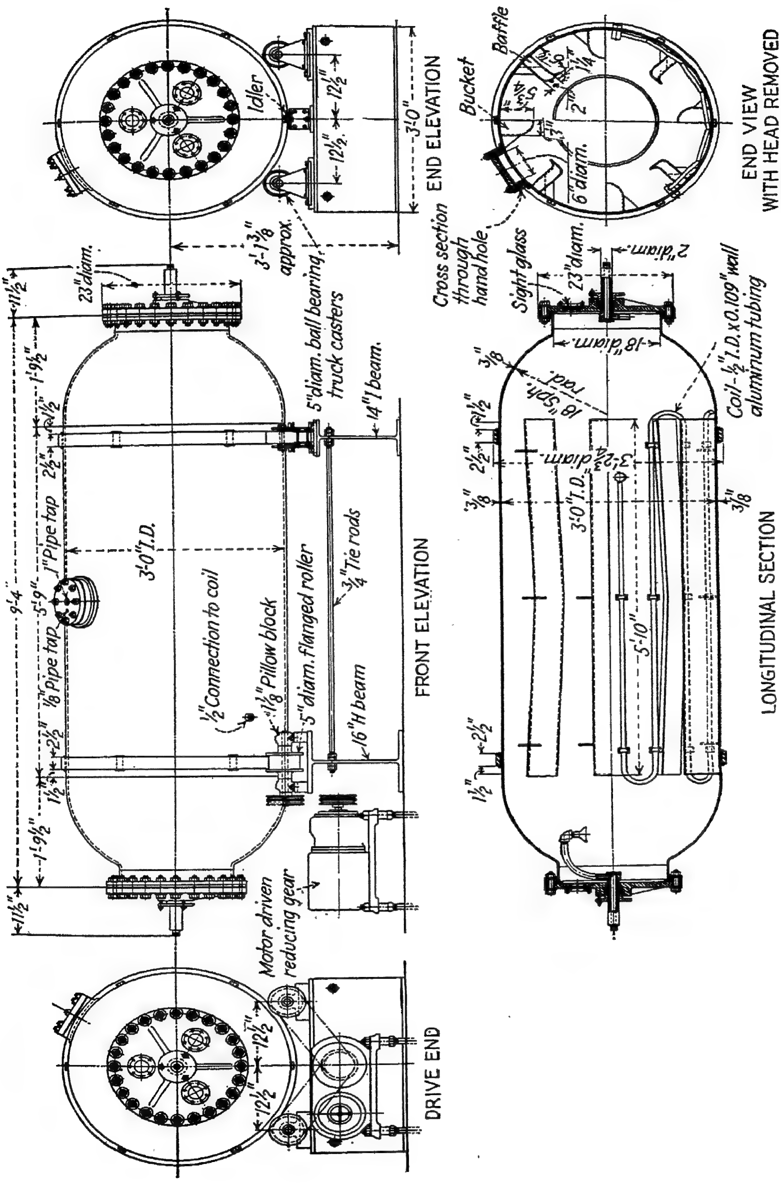


FIG. 46.—Rotary drum fermenter. [Gastrock, Porges, Wells, and Moyer, *Ind. Eng. Chem.*, **30**: 782 (1938).]

Neutralization of Gluconic Acid.—High acidity inhibits the fermentation. The rate at which glucose is converted to gluconic acid is more rapid in the presence of undissolved calcium carbonate than in the presence of free acid. It has been shown by Gastrock and his associates that the use of 26 g. of calcium carbonate per liter of fermentation medium satisfactorily controls the pH of the medium. This quantity of calcium carbonate dissolves readily in the medium and is equivalent to 102.1 g. of gluconic acid per liter. The use of an excess of calcium carbonate retards the fermentation, since the calcium gluconate formed tends to crystallize out and prevent free contact of the medium with the mold.

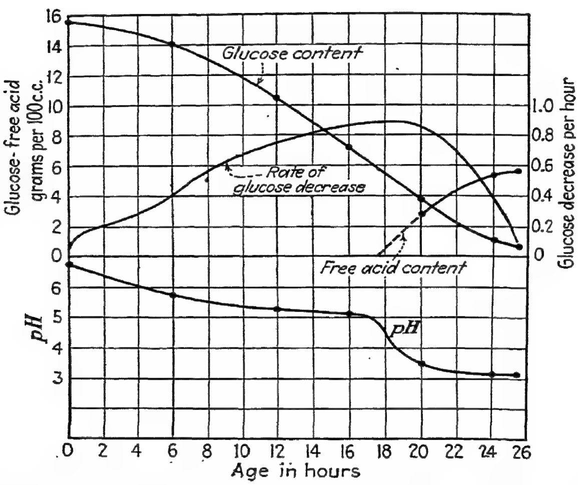


FIG. 47.—Correlation of pH with utilization of glucose (15.6 g. per 100 cc.) by *Aspergillus niger* at 6.0 r.p.m. [*Gastrock, Porges, Wells, and Moyer, Ind. Eng. Chem.*, **30**: 782 (1938).]

The greatest activity in the medium coincides quite closely with the point at which free acid develops. Until the free acid develops, the pH of the medium remains close to 5.5; it then drops rapidly to a value of 3.5 or less. Free acid, with the drop in pH, usually appears about 17 hr. after the start of the fermentation. During the drop in pH, the activity becomes diminished. Thus a pH of 5.5 is favorable for the production of the acid, since the maximum rate of conversion is at pH values of greater than 5. The foregoing figure correlates pH with glucose utilization.

Semicontinuous Production of Gluconic Acid.—A process has been developed by Porges and his associates[1] in which gluconic acid may be

[1] PORGES, N., T. F. CLARK, and E. A. GASTROCK, *Ind. Eng. Chem.*, **32**: 107–111 (1940).

produced successfully on a semicontinuous basis and which possesses several advantages over the single-batch method previously used.

The oxidations were carried out in laboratory- and semiplant-size fermenters, which have already been described, under the following conditions: an air pressure of 30 lb. per sq. in. (gauge), an air flow of 375 cc. per liter of medium per minute, a rotation speed of 9.5 r.p.m. and a temperature of 30°C.

As a result of research, a glucose concentration of approximately 11.5 g. per 100 cc. was found to be optimum, rather than one of 15 g. per 100 cc. The use of lower glucose concentrations resulted in shorter fermentation periods, but in lowered efficiencies of production.

The initial charge of nutrient medium in a fermenter was inoculated with a pregerminated culture. The inoculum for each subsequent charge was secured by floating the mycelia from the previously completely fermented medium. A fermentation was judged to be complete when less than 1 per cent of glucose remained unconverted or when the rate of acid production was negligible in comparison to that of active fermentation. Flotation was accomplished by reducing the pressure in the fermenter to that of the atmosphere for a period of 35 min. During this interval most of the mycelia rose to the upper portions of the medium. Subsequently the lower 80 per cent of the charge was removed, and a new charge of medium was introduced. The upper 20 per cent of the charge retained from the previous fermentation contained most of the active mycelia—more than 85 per cent.

Under the foregoing conditions, a fermentation (excluding the initial one) was usually complete in a little more than 9 hr.

As many as 13 successive fermentations have been carried out, using this general procedure, with no apparent loss in efficiency, according to Porges and his associates.

The advantages of this process are the elimination of the lag period found in the single-batch method through the repeated use of the mycelia, the elimination of the necessity for preparing the pregerminated inoculum for all except the initial charge, and the maintenance of a high rate of oxidation of glucose to gluconic acid.

Gluconic Acid Production by Bacteria.—Many bacteria, though productive of gluconic acid in rather high yields under optimum conditions, are of no practical industrial importance on account of the slowness of the fermentation.

The principal bacteria capable of producing gluconic acid in quantity from glucose belong to the group of acetic acid bacteria. Such acetic acid bacteria as *Acetobacter aceti*, *A. acetosum*, *A. industrium*, and *A. oxydans* may be used. These bacteria are able, under favorable conditions, to oxidize glucose to gluconic acid in the presence of an abundant supply of

oxygen. Currie and Carter,[1] in a patent issued to them in 1933, suggested that glucose concentrations as high as 45 per cent may be used, but 25 per cent is stated to be the optimum concentration. From 0.2 to 2 per cent of mineral salts is added to the fermentation mash to satisfy the mineral requirements of the bacteria. The nutrient glucose solution is permitted to pass in a thin stream down through an apparatus similar to a vinegar generator. The presence of a large amount of oxygen is thus assured. A temperature of 15 to 35°C. is advocated for the fermentation. Hermann,[2] in an Austrian patent, suggested a similar plan for the production of gluconic acid.

Takahashi[3] (1934) advocated the use of *Bacterium Hoshigaki* var. *rosea* and *Bact. industrium* var. *Hoshigaki* in the fermentation of glucose or mannite solutions. Either soybeans or the extract of rice bran is added to the sugar solution to meet the nitrogen requirements of the fermentation organism. The fermentation is permitted to run for 18 days at 26 to 28°C. A very high yield is reported—as high as 103 per cent on the basis of the glucose.

Additional Factors Affecting the Final Yield.—Glucose, mannose, mannite, and maltose may be used as sugars in the production of gluconic acid. Sucrose, lactose, and fructose are not suitable sugars for the fermentation for obvious reasons.

Toluol, potassium cyanide, and carbon monoxide inhibit the formation of gluconic acid when oxygen is available.

If the fermentation bacteria are not grown on nutrient glucose solutions previous to their use in the fermentation mash, the yields of bacteria are much higher.[4]

Uses of Gluconic Acid.—Gluconic acid is used principally as a pharmaceutical. Calcium gluconate is preferred to calcium lactate as a means of supplying calcium to children and to pregnant mothers. The injection of calcium gluconate into cows suffering with milk fever has produced excellent cures.

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[1] U.S. Patent 1,896,811, 1933.

[2] Austrian Patent 133,139, 1933.

[3] U. S. Patent 1,953,694, 1934.

[4] Tanaka, K., *Acta Phytochim* (*Japan*), **7**: 265 (1933).

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CHAPTER XXVII

THE PRODUCTION OF LACTIC ACID BY MOLDS

Recent investigations have indicated that the production of *d*-lactic acid by molds has commercial possibilities and certain apparent advantages.

Historical.—In 1894 the suggestion was made by Eijkmann that the acid formed by *Mucor rouxii* was lactic acid. Chrząszcz confirmed Eijkmann's suggestion in 1901.[1]

Patents were issued to Boullanger (1899) for the production of lactic acid by a mold described as "Lactomyces," an organism now believed to be a species of *Rhizopus*.

Saito reported the production of small quantities of *l*-lactic acid from glucose solutions by *R. chinensis* in 1911.

According to Ehrlich (1919), small quantities of *d*-lactic acid, *l*-malic acid, and succinic acid, but principally fumaric acid, are produced by some species of *Rhizopus*.

Takahashi and his associates (1925) demonstrated that as much as 38 per cent of the glucose fermented was converted to *l*-lactic acid by certain species of *Rhizopus* in some of their experiments. Variable quantities of acetic, formic, fumaric, *l*-malic, and succinic acids and ethyl alcohol were produced at the same time. In later work, Takahashi and Asai (1933) showed that four species of *Mucor* produced traces of lactic acid from glucose media. Traces of pyruvic and succinic acids were likewise found, but ethyl alcohol was the principal product formed.

Miyaji (1930) reported the production of *d*-lactic and succinic acids from glucose media by a new species of *Monilia*, isolated from a commercial culture of soy sauce.

In 1934, results of work carried out by Kanel, in which he used a species of *Rhizopus* similar to *R. japonicus*, were published. Yields of as much as 38 to 40 per cent of lactic acid, on the basis of the carbohydrate consumed, were obtained from invert sugar and from starch. The nutrient medium contained 10 per cent sugar and 4 per cent calcium carbonate. Fumaric acid was occasionally recovered from the older cultures, the quantity formed being a function of the conditions of culture and other factors.

[1] Chrząszcz, T., *Centr. Bakt. Parasitenk.*, Abt. II, **7**: 326 (1901).

Ward and his associates,[1] in studying the biochemical activities of fungi of the genus *Rhizopus*, found several species that converted glucose to *d*-lactic acid in the presence of calcium carbonate. Two strains, *R. oryzae* 394 and *R. oryzae* Went and Geerligs 395, demonstrated particularly good results, yields up to 62 per cent, or greater, being obtained by a surface-culture method.

Methods of Production.—In general, *d*-lactic acid may be produced by selected strains of molds by a surface-culture method or by a rotary-fermenter method. The research of Ward, Lockwood, Tabenkin, and Wells indicates the superiority of the rotary-fermenter process over the surface-culture process.

The following descriptions are based on the research carried out by scientists of the Industrial Farm Products Research Division, Bureau of Chemistry and Soils, U.S. Department of Agriculture.

Surface-culture Method.—In this method, the molds are grown in Pyrex Erlenmeyer flasks, each of which contains 75 cc. of nutrient glucose medium. Sterilized calcium carbonate is added to the flasks at the rate of 4 or 5 g. per flask at the time of inoculation, for in the absence of calcium carbonate only a small amount of acid is formed.

The Mold.—Lactic acid has been produced from the following molds: *Rhizopus arrhizus*, *R. chinensis*, *R. pseudochinensis*, *R. elegans*, *R. oryzae*, *R. salebrosus*, *R. shanghaiensis*, *R. stolonifer*, *R. tritici*, certain Mucors and at least one Monilia. Of the foregoing molds, *R. oryzae* Went and Geerligs 395 and *R. oryzae* 394 are outstanding in their ability to produce *d*-lactic acid. The authors have found no records indicating the production of lactic acid by aspergilli, penicillia, or fusaria.

Preparation of the Mold Suspension.—Lockwood, Ward, and May[2] prepared their mold suspension in the following manner: The mold was grown on sterile moist bread for at least 4 days at 30°C. in order to produce an abundance of spores. Stolons and sporangiospores were placed in a tube or bottle containing sterile water and shaken vigorously to liberate the spores. Using aseptic precautions, the stolon mass was then removed. An estimate of the spore content of the suspension was made next, and the size of the inoculum regulated to provide 12.5 to 100 million spores per 200-cc. Pyrex Erlenmeyer flask containing 75 cc. of nutrient glucose medium.

The Medium.—The composition of the medium used for the production of lactic acid was as shown in the table on page 397.

[1] Ward, G. E., L. B. Lockwood, O. E. May, and H. T. Herrick, *Jour. Am. Chem. Soc.*, **58**: 1286 (1936).

[2] Lockwood, L. B., G. E. Ward, and O. E. May, *Jour. Agr. Research*, **53**: 849 (1936).

TABLE 76.—THE EFFECT OF DIFFERENT SOURCES OF NITROGEN[1] ON THE METABOLISM OF *R. oryzae*[2]

Nitrogen source	Quantity per liter, grams	Weight of mycelium, grams	Glucose consumed, grams	Calcium dissolved			*d*-Lactic acid		Fumaric acid	
				Total, grams	Due to *d*-lactic acid, per cent	Due to fumaric acid, per cent	Weight, grams	Weight yield,[3] per cent	Weight, grams	Weight yield,[3] per cent
$NaNO_3$	3.00	0.000	0.0	0.00	0.0	0.0	0.00	0.0	0.000	0.0
$NaNO_2$	2.56	0.026								
NH_4NO_3	2.88	0.596	8.1	1.05	86.0	3.2	4.03	49.8	0.099	1.2
$(NH_4)_2SO_4$	2.36	0.301	6.2	0.79	95.0	3.5	3.36	54.2	0.082	1.3
NH_4Cl	1.98	0.370	7.2	1.05	87.5	...	4.13	57.4		
Urea	2.14	0.613	8.6	1.24	87.5	6.0	5.44	63.3	0.217	2.5
d,l-Alanine	3.30	0.350	8.3	1.26	86.0	8.6	4.87	58.7	0.312	3.8
d-Glutamic acid	5.46	0.356	7.1	1.08	83.0	6.6	4.05	57.0	0.206	2.9
Glycine	2.67	0.257	5.9	0.87	86.0	7.2	3.37	57.1	0.183	3.1
Peptone	3.24	0.219	8.3	1.29	85.0	5.0	4.95	59.6	0.189	2.3

[1] 75 cc. of 15.1 per cent glucose nutrient solution contained 0.25 g. $MgSO_4 \cdot 7H_2O$ and 0.3 g. KH_2PO_4 per liter; 5 g. $CaCO_3$ per flask. Duration, 14 days. Temperature, 30°C.

[2] LOCKWOOD, L. B., C. E. WARD, and O. E. MAY, *Jour. Agr. Research*, **53**: 849 (1936).

[3] Grams of acid produced divided by grams of glucose consumed.

Glucose (commercial grade)	15 per cent
KH_2PO_4	0.3 g. per liter
NH_4NO_3, $(NH_4)_2SO_4$, or urea, to yield	0.5 g. nitrogen per liter
$MgSO_4 \cdot 7H_2O$	0.25 g. per liter

Effect of Variations in the Constituents of the Medium.—Lockwood, Ward, and May studied the physiology of *R. oryzae* Went and Geerligs[1] and brought out many important facts. The optimum concentration of glucose was 15 per cent, determined on the basis of lactic acid formed, the glucose consumed, and the weight of the mycelia.

The best yields of lactic acid were produced when the concentration of KH_2PO_4 was 0.6 g. per liter. Doubling this amount of the salt did not increase the yield of lactic acid.

Ammonium chloride; ammonium nitrate; ammonium sulphate; *d-l*-alanine; *d*-glutamic acid; glycine; peptone; and urea served as satisfactory sources of nitrogen. Sodium nitrate failed to produce growth, while sodium nitrite produced only a slight amount of growth. Table 76 shows the effect of different sources of nitrogen on the metabolism of *R. oryzae.*

Effect of Zinc Sulphate.—When calcium carbonate was a constituent of the culture medium, the addition of 10 mg. of zinc, in the form of zinc sulphate, caused an increase in the weight of mycelium, in the glucose consumption, and in the absolute quantity of lactic acid formed by *R. oryzae* Went and Geerligs 395. The yield of *d*-lactic acid, calculated in terms of grams of acid formed divided by grams of glucose utilized, became progressively smaller as the concentration of zinc was increased, however. In the absence of calcium carbonate, there was no increase in the glucose consumption.

Effect of Other Salts.—Ferric and chromium ions, both in the presence and absence of calcium carbonate, produced no apparent effect on glucose consumption or lactic acid production.

Temperature.—A temperature of 30°C. is favorable for the production of lactic acid. At 40°C., the growth of the mold is more rapid but the yield of acid is less than at 30°C.

Incubation Period.—Cultures are usually incubated for 16 to 21 days. The effect of the incubation period on results obtained with *R. oryzae*[2] is shown in Table 77.

Fumaric Acid.—Production of fumaric acid by *R. oryzae* Went and Geerligs 395 is a variable factor. For example, Lockwood, Ward, and May found that a temperature of 40°C. is more favorable for its production than 30°C.; that ammonium nitrate (NH_4NO_3) in a concentration

[2] Lockwood, L. B., C. E. Ward, and O. E. May, *Jour. Agr. Research,* **53**: 849 (1936).

greater than 6 g. per liter and that high concentrations of glucose inhibit its formation; and that it is not produced during the early part of the fermentation but only after several days.

TABLE 77.—THE EFFECT OF THE LENGTH OF THE INCUBATION PERIOD ON THE METABOLISM OF *R. oryzae*[1]

Age, days	Weight of mycelium, grams	Glucose consumed, grams	Calcium dissolved			*d*-Lactic acid		Fumaric acid	
			Total, grams	Due to *d*-lactic acid, per cent	Due to fumaric acid, per cent	Weight, grams	Weight yield,[2] per cent	Weight, grams	Weight yield,[2] per cent
5	0.040	1.3	0.067	..	....	0.060			
9	0.039	2.0	0.24	87	0	0.93	46.5	0	0
13	0.220	6.3	0.85	88	0	3.36	53.3	0	0
17	0.889	10.5	1.28	78	8.7	4.50	42.9	0.314	3.0
21	1.032	11.2	1.44	72	13.9	4.66	41.6	0.564	5.0

[1] 75 cc. of 15 per cent glucose; 0.25 g. $MgSO_4 \cdot 7H_2O$, 0.3 g. KH_2PO_4, and 2.88 g. NH_4NO_3 per liter; 5 g. $CaCO_3$ per flask. Temperature, 30°C.

[2] Grams of acid produced divided by grams of glucose consumed.

Other End Products.—Acetic, *l*-malic, and succinic acids and ethyl alcohol have been detected in traces, or in small quantities, in some glucose media fermented by *R. oryzae.*

Yields.—Yields of as high as 62 to 67 per cent lactic acid have been obtained by the surface-culture method on the basis of the sugar consumed.

The largest quantity of *d*-lactic acid is produced just before sporulation of the mold.

The Rotary-fermenter Process.—Lactic acid is produced in 30 to 35 hr.[1] in rotating aluminum drums of the type described in the chapter on the gluconic acid fermentation. This process is superior to the surface-culture process both from the standpoint of the shorter fermentation period and of the quantity of acid produced from the sugar consumed, yields of 70 to 75 per cent of acid being common.

Procedure.—Spores are produced for inoculation purposes by growing the mold on sterile moist bread. A special germination medium is inoculated to contain 420 million spores per 1.5 liters of the medium. The germination medium containing the spores is shaken for 24 hr. at 30°C. in a 4-liter glass bottle equipped with an outlet tube. By using this medium, the composition of which is shown in Table 78, a saving in fermentation time is effected.

A 3-liter portion of the fermentation medium is inoculated with 250 cc. of the germinated spore culture and placed in the aluminum drum. The

[1] WARD, G. E., L. B. LOCKWOOD, B. TABENKIN, and P. A. WELLS, *Ind. Eng. Chem.*, **30**: 1233 (1938).

drum is operated at a gauge pressure of 5 lb. per sq. in. and a rotation speed of 13 r.p.m. Air, measured at the exit, flows through at the rate of 150 cc. per min., while the temperature is maintained at 35°C.

TABLE 78.—SOME MEDIA USED IN LACTIC ACID PRODUCTION[1]

Substance	Germination medium, grams	Fermentation medium, grams
Glucose (91.5 per cent, commercial)...	110	150
Urea..................................	2.0	2.0
KH_2PO_4............................	0.60	0.60
$MgSO_4 \cdot 7H_2O$..................	0.25	0.25
$ZnSO_4 \cdot 7H_2O$..................	0.088	0.044
$CaCO_3$..............................	10.0	*
Octadecyl alcohol.....................		0.03†
Distilled water.......................	to 1 liter	to 1 liter

[1] WARD, G. E., L. B. LOCKWOOD, B. TABENKIN, and P. A. WELLS, *Ind. Eng. Chem.*, **30**: 1233 (1938).

* 200 g. to each 3-liter portion—sterilized separately.

† Dissolved in 1.7 cc. ethanol (added to prevent excessive foaming of the fermentation medium during the rotation of the drum).

On account of the limited solubility of calcium lactate and in view of the higher yields obtained by this method, it is necessary to use a somewhat lower concentration of glucose than is the case in the surface-culture method. When a precipitate of calcium lactate settles out, forming a white mass, fermentation is much inhibited. If the settling occurs before all the sugar is utilized, it is particularly objectionable. By using 13 per cent, or less, glucose, satisfactory results are usually obtained.

Results of a Typical Experiment.—The following data are quoted to illustrate the result of a typical fermentation carried out by the rotating-drum method, using *R. oryzae* Went and Geerligs:

	Grams per 100 Cc.[1]
Original glucose concentration..............................	13.3
Glucose consumed..	12.8
Ethyl alcohol produced..	0.62
Calcium in solution...	2.245
Lactic acid equivalent to dissolved calcium...................	10.12
Lactic acid found by analysis.................................	9.66
Acidity due to lactic acid, per cent..........................	95.4
Yield of *d*-lactic acid, based on glucose consumed, per cent....	75.5

[1] WARD, G. E., L. B. LOCKWOOD, B. TABENKIN, and P. A. WELLS, *Ind. Eng. Chem.*, **30**: 1233 (1938).

The rate at which glucose was consumed in this experiment is indicated in Fig. 48 on page 400.[1] It will be observed that there was but

[1] WARD, LOCKWOOD, TABENKIN, and WELLS, *loc. cit.*

little consumption of glucose during the first 15 hr., but during the rest of the experiment the sugar was consumed at a fairly rapid rate.

Respiration and Lactic Acid Production.—Waksman and Foster[1] have carried out research concerning respiration and lactic acid production by a species of the genus *Rhizopus*. They have suggested reactions to explain the mechanism of these important processes.

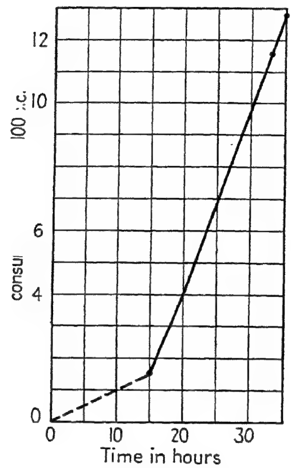

FIG. 48.—Course of a typical *d*-lactic acid submerged fermentation induced by *Rhizopus oryzae*. [*Ward, Lockwood, Tabenkin, and Wells, Ind. Eng. Chem.*, **30**: 1233 (1938).]

Advantages of Mold Process.—Although *d*-lactic acid, a physiologically important acid (known as sarcolactic acid, also) is not produced commercially by molds at present, this process has, as pointed out by Ward and his associates, several apparent advantages. Owing to the fact that urea is used as the source of nitrogen, a lactic acid free from color and of relatively high purity may be produced. The fermentation is rapid, and salts of lactic acid are easily recovered. Furthermore, this process provides a source of *d*-lactic acid, which may be used for the preparation of crystallized *d*-lactic acid.

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[1] WAKSMAN, S. A., and J. W. FOSTER, *Jour. Agr. Research*, **57**: 873 (1938).

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LOCKWOOD, L. B., G. E. WARD, and O. E. MAY: The Physiology of *Rhizopus oryzae*, *Jour. Agr. Research*, **53**: 849 (1936).

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WAKSMAN, S. A., and J. W. FOSTER: Respiration and lactic acid production by a fungus of the genus *Rhizopus*, *Jour. Agr. Research*, **57**: 873–900 (1938).

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WARD, G. E., L. B. LOCKWOOD, O. E. MAY, and H. T. HERRICK: Studies in the Genus *Rhizopus*. I. The Production of Dextro-lactic Acid, *Jour. Am. Chem. Soc.*, **58**: 1286 (1936).

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Some Patents

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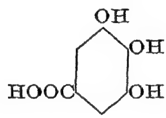
WARD, G. E., L. B. LOCKWOOD, and O. E. MAY: Fermentation Process for the Manufacture of Dextro-lactic Acid, U.S. Patent 2,132,712, Oct. 11, 1938.

CHAPTER XXVIII

THE GALLIC ACID FERMENTATION

Gallic acid (trihydroxybenzoic acid) has the following structural formula:



It occurs naturally in gall nuts, sumac, walnuts, tea, and other plants. It is prepared chemically by the hydrolysis of tannin.

Scheele discovered gallic acid when he was studying the effect of a mold on a water infusion of gall nuts (1787, or earlier). Gall nuts are rich in tannin compounds and are produced principally on species of the oak tree and the sumac, as the result of insect injuries.

Van Tieghem (1867) carried out classical studies in connection with the gallic acid fermentation. (The interested reader should refer to his early publications. To Van Tieghem, credit is due for being the first man to establish the importance of the aspergilli in the biochemical field, his work having been done at a time when very little was known of modern pure-culture methods.) Van Tieghem identified *Aspergillus niger* as the predominating mold in the fermentation. The *Penicillium glaucum* group of molds also possessed the ability to ferment tannin to gallic acid. Van Tieghem showed that air was essential for mold growth and for the successful production of gallic acid from moist gall nuts or tannin liquor.

In one of the oldest methods used for the production of gallic acid by fermentation, the substances containing tannin were piled up in heaps and moistened with water. Molds developed throughout the heap, which was stirred occasionally and maintained at a temperature of approximately 30°C. After a fermentation period of about a month the gallic acid was leached from the heap.

Present-day methods make use of clear tannin extracts, which are sterilized and then inoculated with pure cultures of a species of *Aspergillus*. The solution is agitated by mechanical means, and air is blown through it. The temperature of the fermentation is carefully controlled. Analysis of the mash is made occasionally in order that the fermentation may be stopped promptly after the tannin has been completely utilized.

The conversion of tannin to gallic acid is actually brought about by means of the enzyme "tannase." Fernbach and Pottevin, each working independently, demonstrated that *A. niger* produced tannase in the presence of tannin and nutrient materials. Using the mold-free enzyme, it was shown that the fermentation would proceed independently of the mold.

Knudson discovered that he could cause a progressive increase in the tannase content of *A. niger* by replacing the sugar in Czapek's solution with tannic acid. He obtained a maximum production of tannase when the 10 per cent of sugar in the solution had been replaced with 2 per cent of tannic acid.

Gallic acid has several important uses in industry. It has been used in the manufacture of gallocyanin, a dye; it is used as a basic material in the production of alizarin brown. Inks are made from it. Condensation with sulphuric acid yields hexahydroxyanthraquinone. In combination, gallic acid finds use as a skin remedy.

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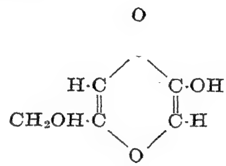
———: Sur la fermentation gallique, *Compt. rend.*, **65**: 1091–1094 (1867).

CHAPTER XXIX

THE KOJIC ACID FERMENTATION

Kojic acid may be produced readily by biological means, but at the present time this acid is of theoretical interest only, in spite of the fact that derivatives may be produced without particular difficulty from it.

Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) is represented by the following chemical formula:



Historical.—Saito[1] (1907) first isolated the acid as a by-product of the fermentation of steamed rice by *Aspergillus oryzae*. When ferric chloride was added to an aqueous solution of the acid, an intense red color developed. A Japanese biochemist, Yabuta,[2] investigated the acid isolated by Saito and named it "kojic acid" (1912). The constitution of the acid was established by Yabuta[3] in 1924.

Traetta-Mosca[4] (1914) showed that kojic acid was produced by *A. glaucus* from sucrose, glucose, fructose, and glycerol. Wijkman[5] obtained the acid by the fermentation of sucrose using an unnamed strain of *Aspergillus*. Three years later, Kinoshita[6] indicated that the use of cobaltamines as sources of nitrogen caused increased yields of kojic acid from sucrose when using *A. oryzae*. When purpureo-cobaltic chloride was added to a 10 per cent sucrose mash in a 0.5 per cent concentration, a 33 per cent yield by weight of kojic acid was obtained in 25 days.

In 1927, Tamiya[7] pointed out that several different aspergilli had the ability to produce kojic acid from sucrose. Among the molds mentioned

[1] SAITO, K., *Botan. Mag. Tokyo*, **21**: 240 (1907).
[2] YABUTA, T. Refer to the bibliography at the end of the chapter.
[3] YABUTA, T., *Jour. Chem. Soc.*, **125**: 575 (1924).
[4] TRAETTA-MOSCA, F., *Ann. chim. applicata*, **1**: 477 (1914).
[5] WIJKMAN, N., *Zeit. physiol. Chem.*, **132**: 104 (1924).
[6] KINOSHITA, K., *Acta Phytochim. (Japan)*, **3**: 31 (1927).
[7] TAMIYA, H., *Acta Phytochim. (Japan)*, **3**: 51 (1927).

were *A. oryzae; A. flavus*, var.; *A. gymnosardae; A. awamori; A. candidus; A. clavatus; A. fumigatus;* and *A. giganteus.*

In 1929, Challenger, Klein, and Walker[1] published the results of research which showed that kojic acid could be produced from xylose by *A. oryzae.* During the same year Katagiri and Kitahara[2] reported on the formation of kojic acid from pentoses, gluconic acid, and other substances.

Since 1929, several papers have been published concerning various aspects of the kojic acid fermentation. Titles to some of the more significant papers will be found in the reference list at the end of the chapter.

Microorganisms Producing Kojic Acid.—Several molds of the genus *Aspergillus* have the ability to produce kojic acid from suitable carbon-containing nutrient solutions. In addition to the species enumerated in the foregoing paragraphs, the following molds produce kojic acid: *A. albus, A. effusus, A. nidulans, A. parasiticus, A. tamarii*, and *Penicillium daleae.* From among the bacteria, several species of *Acetobacter* may also form kojic acid under favorable conditions.

Carbon Sources.—A fairly large number of carbon-containing substances have been fermented with the production of kojic acid by different microorganisms. These substances include starches; dextrins; disaccharides, such as sucrose and maltose; the hexoses—glucose, fructose, mannose, and galactose; the pentoses—xylose and arabinose; and sorbitol, dulcitol, *i*-adonitol, inulin, inositol, glycerol, glycero-beta-phosphate, dihydroxyacetone, gluconic acid, tartaric acid, and other substances. The best yields have, in general, been obtained from glucose and xylose.

Concentration of Carbon-containing Substance Used.—The concentrations of carbon-containing materials used have been varied from approximately 5 to 30 per cent. May and his associates[3] used sugar concentrations varying from 15 to 33 per cent in their work. They obtained highest yields with *A. flavus* when using a concentration of 20 per cent glucose. Barham and Smits[4] found a 15 per cent concentration of xylose to be most suitable for fermentation. Katagiri and Kitahara used 5 per cent concentrations of a large number of substances with satisfactory results in most cases. The same concentration has been used by other workers.

Nutrient Salts.—The types of nutrient salts used for kojic acid production are illustrated in the paragraphs following.

[1] Challenger, F., L. Klein, and T. K. Walker, *Jour. Chem. Soc.*, p. 1498 (1929).

[2] Katagiri, H., and K. Kitahara, *Bull. Agr. Chem. Soc. (Japan)*, **5**: 38 (1929).

[3] May, O. E., A. J. Moyer, P. A. Wells, and H. T. Herrick, *Jour. Am. Chem. Soc.*, **53**: 774 (1931).

[4] Barham, H. N., and B. L. Smits, *Ind. Eng. Chem.*, **28**: 567 (1936).

May and his coworkers[1] obtained satisfactory results when using the following salts in the concentrations recorded:

	Grams per Liter of Glucose Solution
$MgSO_4 \cdot 7H_2O$	0.500
KCl	0.100
H_3PO_4	0.054
NH_4NO_3	1.125

The medium of Kinoshita,[2] often referred to as "medium *K*," is of the following composition:

	Grams in 1,000 Cc. of Water
$MgSO_4 \cdot 7H_2O$	0.5
KH_2PO_4	1.0
NH_4NO_3	0.4

Katagiri and Kitahara used media containing 0.01 per cent $MgSO_4 \cdot 7H_2O$, 0.1 per cent KH_2PO_4, 0.05 per cent $(NH_4)_2SO_4$ and 0.01 per cent $CaCl_2$ with 5 per cent of carbon-containing substance.

Kojic acid may be produced in the usual Czapek-Dox medium.

Ammonium nitrate is very satisfactory as a source of nitrogen. Apparently better yields are obtained when the concentration of the salt is small, but sufficiently large to permit growth of the mold.

pH.—The optimum pH for the production of kojic acid under a given set of conditions must be determined by experimentation if this fact is not already known. The range of pH 2 to 5 or above has been used by various workers. Katagiri and Kitahara employed an initial pH of 5.0 to favor the growth of *A. oryzae* but found that a pH of 2.4 stimulated formation of kojic acid. A pH of 5.5 was found to be optimum for the fermentation of sucrose by Tamiya. Barham and Smits obtained highest yields when using a pH range of 2 to 3.5 in the fermentation of xylose by *A. flavus*. These men advanced the opinion that the optimum pH for the fermentation was the lowest one that the organism would tolerate. (Compare with the citric acid fermentation.)

The addition of calcium carbonate to a kojic acid fermentation results in a greatly diminished yield of the acid. It is believed that the decreased yield is due to a change in the pH of the mash.

Effect of Added Substances.—In a survey of 40 organic compounds, May and his associates[3] found that ethylene chlorhydrin in a concentration

[1] May, O. E., A. J. Moyer, P. A. Wells, and H. T. Herrick, *Jour. Am. Chem. Soc.*, **53**: 774 (1931).

[2] Kinoshita, K., *Acta Phytochim.* (*Japan*), **3**: 31 (1927).

[3] May, O. E., G. E. Ward, and H. T. Herrick, *Centr. Bakt. Parasitenk.*, Abt. II, **86**: 129 (1932).

of 100 mg. per liter produced a marked increase in the yield of kojic acid in a period of 10 days.

Oxalic, citric, formic, hydrochloric, and nitric acids inhibit the formation of kojic acid by fermentation.[1]

Temperature.—The temperature range of 29 to 35°C. is optimum for the fermentation. May and his associates advocated a temperature of 30 to 35°C. for *Aspergillus flavus* (a strain of the *A. flavus-oryzae* group, secured from Dr. Thom as culture number 3538). A temperature of 29 to 31°C. was used by Kitahara and Katagiri; one of 35°C. by Barham and Smits; and one of 20°C. by Gould. Sometimes the temperature may be dropped to 25°C. or lower after the fermentation has proceeded at a higher temperature for about 5 days, resulting in increased yields.

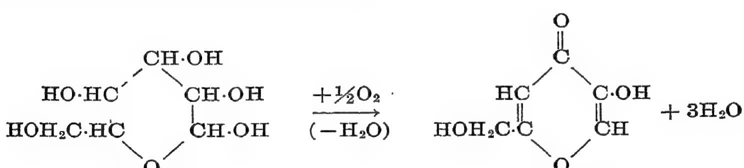
Duration of Fermentation.—The fermentation generally requires 9 to 20 days for completion, the period depending on the type of substrate, species of mold, the temperature, pH, and other factors. After the sugar has been consumed, the kojic acid may be utilized by the mold, resulting in decreased yields.

Yields.—Yields amounting to 50 to 60 per cent may be produced from glucose solutions by *A. flavus*. Table 79 illustrates the nature of the yields obtained from various compounds by Katagiri and Kitahara.

Other details concerning the fermentation may be ascertained by reference to some of the original papers cited at the end of this chapter.

Mechanism of Kojic Acid Formation.—There have been several schemes proposed to explain the production of kojic acid by fermentation. Some of these will be considered briefly.

Yabuta held that kojic acid arose directly from glucose by a simple process of oxidation and dehydration:



Glucose Kojic acid

Kinoshita (1927) and Haworth (1928) offered a similar explanation for the origin of kojic acid. The basis for this theory appears to be that glucose yields relatively large quantities of the acid in comparison with some of the other carbon-containing materials. This theory does not explain how kojic acid is formed from compounds containing less than 6 carbon atoms, such as the pentoses, glycerol, and dihydroxyacetone, or from 7-carbon compounds.

[1] Barham, H. N., and B. L. Smits, *Trans. Kansas Acad. Sci.*, **37**: 91 (1934).

TABLE 79.—AMOUNT OF KOJIC ACID PRODUCED BY *A. oryzae*[1]
(From 100 cc. of 20 days' culture containing 5 g. of substance)

Substance	Kojic acid		Observed by Sakaguchi (1932)	Observed by Tamiya (1932)
	Grams in 100 cc.	Yield on basis of sugar present, per cent		
Glycogen	0	0	..	0
Inulin	0.65	14.8	..	+
Sucrose	1.45	34.8	+	+
Maltose	1.70	40.8	..	+
Lactose	±	±	+	0
Trehalose	0.095	2.3	..	+
Glucose	0.75	18.1	+	+
Fructose	0.40	10.2	+	+
Mannose	0.23	5.8	..	+
Galactose	0.04	1.0	+	0
α-Methylglucoside	0	0	..	0
Rhamnose	0	0	+	0
Arabinose	0.08	2.0	+	+
Xylose	0.40	10.2	+	+
Dihydroxyacetone[2]	0.52	33.0	+	0
Glyceraldehyde	±	±		
Methylglyoxal	0	0		
Inositol[3]	0.04	0.9	..	+
Mannitol	±	±	+	±
Sorbitol[3]	0.4	10.2	..	+
Dulcitol[3]	0.4	10.2	..	+
Erythritol	±	±	+	+
Glycerol	0.16	4.2	+	±
Na-glycerophosphate	+	+		
Ethylene glycol	0	0	..	0
Ethyl alcohol	0	0	+	
Methyl alcohol	0	0	±	
Gluconic acid[4]	0.34	9.4	±	0
Saccharic acid	0	0	..	0
Lactobionic acid	±	±		
Arabonic acid	0	0		
Glyceric acid	0	0		
Succinic acid	0	0	..	±
Tartaric acid	0	0	..	0
Oxalic acid	0	0		
Acetic acid	0	0	±	0
Pyruvic acid	0	0	..	0
Lactic acid	0	0	..	0
Glycolic acid	0	0	..	0

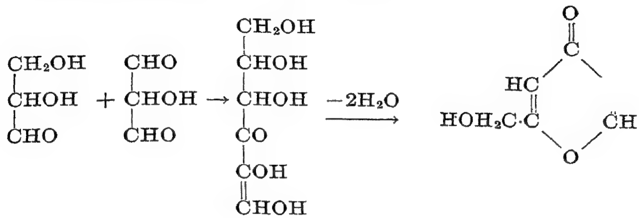
[1] KATAGIRI, H., and K. KITAHARA, *Mem. Coll. Agr., Kyoto Imp. Univ.*, No. 26 (Chem. Series 15) March, 1933.

[2] The mixture of 2 per cent dihydroxyacetone and 3 per cent α-methylglucoside is used for the observation with dihydroxyacetone.

[3] Incubation for 14 days.

[4] Incubation for 40 days.

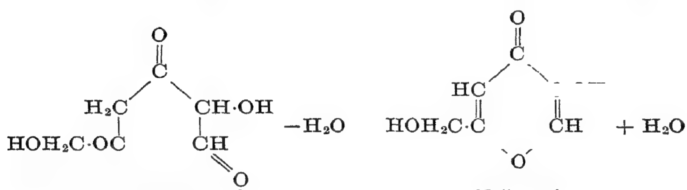
Corbellini and Gregorini[1] (1930) advanced the suggestion that a pyrone nucleus is synthesized from 3-carbon compounds. For example, 2 molecules of 3-carbon compounds may condense to form a molecule that by dehydration passes to kojic acid:



Kojic acid

Pyrones are readily formed chemically from acyclic polyketones through dehydration.

May and his associates share the view advanced by Corbellini and Gregorini that kojic acid is synthesized from some substance containing 2 or 3 carbon atoms. They suggest that 1-hydroxyacetyl-3-formyl-3-hydroxyacetone may be the precursor of kojic acid, although the former compound has not been isolated from a kojic acid fermentation:



1-Hydroxyacetyl-3-formyl-3-hydroxyacetone   Kojic acid

Birkinshaw, Charles, Lilly, and Raistrick[2] (1931) have proposed two theories to explain the mechanism of kojic acid formation. One theory suggests the formation of kojic acid through the condensation of acetaldehyde. This theory is based upon the fact that ethyl alcohol is frequently found in mold fermentations, especially in those in which kojic acid is one of the end products. The suggestion is also made that the presence of ethanol presupposes the occurrence of acetaldehyde as a precursor. Katagiri and Kitahara (1929) showed that acetaldehyde could be detected by sulphite fixation in several different types of media fermented by *Aspergillus oryzae* (Higuchi blue). Sakaguchi found that ethanol

[1] Corbellini, A., and B. Gregorini, *Gazz. chim. ital.*, **60**: 244 (1930).

[2] Birkinshaw, J. H., J. H. V. Charles, C. H. Lilly, and H. Raistrick, *Trans. Roy. Soc. London*, **B220**: 127 (1931).

increased the yield of kojic acid from glucose solutions. He therefore believed that ethanol might be an intermediate in the fermentation.

The addition of fixing agents, such as sulphite and dimedon, to mashes being fermented by *A. tamarii* did not prevent kojic acid formation, nor could fixation products be isolated from the media, according to Gould.[1]

Katagiri and Kitahara (1933) have reported that no kojic acid could be detected in media that contained calcium hexosediphosphate or calcium and magnesium hexosemonophosphates. Furthermore, these investigators were unable to obtain kojic acid from media containing acetaldehyde; acetone; or pyruvic, parapyruvic, or acetoacetic acids.

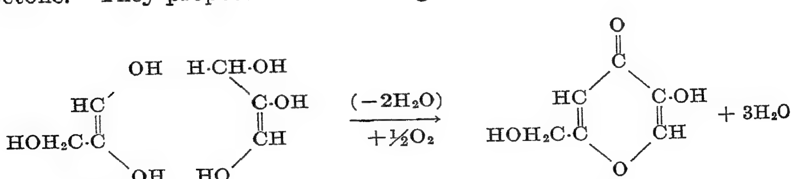
Thus, although acetaldehyde and ethanol are produced in some mold fermentations, there is no direct evidence to show that they are intermediate products in the formation of kojic acid.

The second theory proposed by Birkinshaw and his associates was that a reserve carbohydrate would be anabolized from the carbon-containing substance in the medium and that subsequently the reserve carbohydrate would be hydrolyzed to a compound that could be converted to kojic acid.

Research carried out by Gould has indicated that kojic acid is probably not produced from reserve carbohydrates. Gould grew mycelial mats of *A. tamarii* on media containing several different carbon-containing substances. The mats were washed free of kojic acid, dried, and then ground to a fine powder. The powder was substituted for sugar in the Czapek-Dox medium. No kojic acid was produced in 90 days.

Tamiya proposed that from the various constituents of the medium a hexose might be synthesized which would give rise to kojic acid.

In 1931, Challenger, Klein, and Walker reported that *A. oryzae* produced a yield of more than 30 per cent of the theoretical from dihydroxyacetone. They proposed the following scheme:



Dihydroxyacetone

Kojic acid

Challenger and his associates thus assumed that dihydroxyacetone or glyceraldehyde were the logical intermediate products in the formation of kojic acid.

[1] Gould, B. S., *Biochem. Jour.*, **32**: 797 (1938).

Katagiri and Kitahara (1933) concluded, as the result of extensive research, that dihydroxyacetone would be the most probable substance of those suggested to assume an important role in kojic acid formation.

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CHAPTER XXX

THE FUMARIC ACID FERMENTATION

Fumaric acid (HOOC·CH=CH·COOH), an unsaturated acid, is produced by several molds, principally species of the genus *Rhizopus.* Other genera of the family *Mucoraceae*, namely, *Circinella*, *Cunninghamella*, and *Mucor*,[1] also have the ability to produce fumaric acid from nutrient sugar solutions. At least one species of *Aspergillus* and one of *Penicillium* have been credited with the production of this acid.

Ehrlich[2] (1911) first reported on the formation of fumaric acid by molds.

There is much variation in acid production even in strains that appear to be identical morphologically. For example, Foster and Waksman[3] have reported the case of acid production by races of a strain of *Rhizopus nigricans*. The female race produced fumaric acid from a nutrient glucose solution, while the male race failed to produce fumaric acid under the same and a variety of conditions. These results were not characteristic of sexual pairs in general.

Ordinarily, molds produce only small quantities or traces of the acid, but one strain of *R. nigricans* has the ability to convert 40 to 50 per cent of the sugar consumed to fumaric acid.

Other acids, for example, lactic acid, are frequently produced simultaneously with fumaric acid (see Chap. XXVII).

Considerable information concerning the fumaric acid fermentation may be obtained through a study of the papers cited at the end of this chapter. Birkinshaw[4] (1937) and Lockwood and Moyer[5] (1938) have very briefly reviewed some of the literature concerning the production of fumaric acid by molds. Foster and Waksman have carried out extensive research concerning fumaric acid production by the genus *Rhizopus*, with special reference to the effect of zinc on growth and acid production.

In the following table, some data concerned with molds that produce fumaric acid are summarized.

[1] Foster, J. W., and S. A. Waksman, *Jour. Am. Chem. Soc.*, **61**: 127 (1939).
[2] Ehrlich, F., *Ber.*, **44**: 3737 (1911).
[3] Foster, J. W., and S. A. Waksman, *Science*, **89**: 37 (1939).
[4] Birkinshaw, J. H., *Biol. Rev.*, **12**: 357 (1937).
[5] Lockwood, L. B., and A. J. Moyer, *Bot. Rev.*, **4**: 140 (1938).

TABLE 80.—SOME MOLDS PRODUCING FUMARIC ACID[1]

Molds	Investigators	Year research reported
Aspergillus fumaricus...............	Wehmer[21,22]	1918, 1928
A. fumaricus.......................	Thies[18]	1930
Circinella sp......................	Foster and Waksman[9]	1939
Cunninghamella sp..................	Foster and Waksman[9]	1939
Mucor sp...........................	Foster and Waksman[9]	1939
M. stolonifer (*R. nigricans*)..........	Ehrlich[6,7]	1911, 1919
M. stolonifer (*R. nigricans*)..........	Butkewitsch & Federoff[3,4,5]	1929, 1930
M. stolonifer (*R. nigricans*)..........	Gottschalk[11]	1926
Penicillium griseo-fulvum Dierckx.....	Raistrick & Simonart[14]	1933
Rhizopus japonicus..................	Takahashi, Sakaguchi, and Asai[17	1926, 1927
R. niveus..........................	Takahashi and Sakaguchi[16]	1925
R. oryzae..........................	Ward, Lockwood, May, and	1936
R. oryzae..........	Lockwood, Ward, & May[12]	1936
R. pseudochinensis.	Takahashi and Asai[15]	1925
R. shanghaiensis...	Takahashi and Sakaguchi[16]	1925
R. tritici..........	Takahashi and Sakaguchi[16]	1925

[1] The figures given in the parentheses refer to the bibliography following.

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3. BUTKEWITSCH, W., und M. W. FEDEROFF: Über Bildung von Fumarsäure in den Zuckerculturen von *Mucor stolonifer* (*Rhizopus nigricans*) und sein Verhalten zur Brenztraubensäure, *Biochem. Zeit.*, **206**: 440–456 (1929).
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CHAPTER XXXI

MANNITOL PRODUCTION BY MOLDS

Mannitol ($CH_2OH \cdot (CHOH)_4 \cdot CH_2OH$), an alcohol, occurs naturally in manna, an exudate from certain plants, prominent among which is the manna ash tree, *Fraxinus ornus*. It is formed as a fermentation product from fructose by certain bacteria.

Braconnot (1811) and Vauquelin (1813) reported that mannitol was found to be a constituent of the tissue of some of the higher fungi. Mannitol has since been found in the mycelium of some of the lower fungi as well. As such, it is regarded as a reserve food product rather than as a product of fermentation.

Several fungi, however, produce mannitol as a fermentation product from nutrient sugar solutions. Mannitol is produced from glucose in a Czapek-Dox solution by certain white aspergilli,[1] by *Aspergillus elegans*, by *A. nidulans*, by *Helminthosporium geniculatum*, by a species of *Clasterosporium*, by *Byssochlamys fulva*, and by *Penicillium chrysogenum*. It is produced from sucrose, together with itaconic acid, by *A. itaconicus;* and from glycerol by molds of the *A. glaucus* group. Ethyl alcohol is formed by two of the foregoing fungi, namely, *H. geniculatum* and the species of *Clasterosporium*, as one of the main products.[2] Other products are formed in small quantities by some of these fungi, such as glycerol, acetaldehyde, succinic acid, and malic acid.

Pruess and his associates[3] have isolated mannitol from *A. fischeri* and *A. oryzae*.

Yields of as much as 50 per cent mannitol, based on the sugar utilized, have been produced by a white species of *Aspergillus* (Thom 4640.489) from the Czapek-Dox solution of glucose (2 g. of $NaNO_3$; 1 g. of KH_2PO_4; 0.5 g. of KCl; 0.5 g. of $MgSO_4 \cdot 7\ H_2O$; 0.01 g. $FeSO_4 \cdot 7\ H_2O$; 50 g. of glucose and water to 1,000 cc.).

Two other species of white aspergilli, a strain of *A. elegans* and five strains of *A. nidulans*, were investigated by Birkinshaw and his associ-

[1] Birkinshaw, J. H., J. H. V. Charles, A. C. Hetherington, and H. Raistrick, *Trans. Roy. Soc. London*, **220B**: 153 (1931).

[2] Birkinshaw, J. H., and H. Raistrick, *Trans. Roy. Soc. London*, **220B**: 331 (1931).

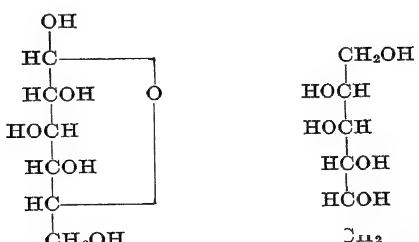
[3] Pruess, L. M., W. H. Peterson, and E. B. Fred, *Jour. Biol. Chem.*, **97**: 483 (1932).

ates.[1] The metabolic solutions were incubated at 23 or 24°C. usually, for periods of 2 to 10 weeks. Control of aeration was found to be important. Higher yields were obtained by restricting the air supply, this being effected by keeping the flasks closed to all air supply except the small amounts of sterilized air that were passed through the flasks during a 30-min. period once a day. Unrestricted aeration resulted in lower yields, for the "water-soluble product" formed was used up as the available glucose disappeared. Some of the highest yields were obtained when the exhausted metabolism solutions were replaced with fresh nutrient glucose solutions. As a result of using the mycelia over again, shorter fermentation periods were required.

The same strain of *Aspergillus*[2] produced yields of about 35 per cent *d*-mannitol from glucose, mannose, and galactose on the basis of the sugar utilized. A smaller yield of *d*-mannitol was obtained from xylose, a pentose. The yield from arabinose was poorer than that from xylose. No mannitol was produced from fructose, which is readily fermented by some bacteria and reduced by chemical means to *d*-mannitol.

Byssochlamys fulva, Olliver and Smith,[3] an ascomycete and a cause of spoilage in processed fruits, produces mannitol from glucose to the extent of about 30 per cent, on the basis of the sugar consumed, when grown in the Czapek-Dox solution. The inoculated solutions were incubated at 24°C. Byssochlamic acid ($C_{18}H_{20}O_6$; m.p. 163.5°C.) was obtained in yields of about 0.5 per cent along with mannitol.

The following equation represents the formation of *d*-mannitol from *d*-glucose:



d-Glucose *d*-Mannitol

The mechanism by which *d*-mannitol is produced from the pentoses is not known at present.

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[1] BIRKINSHAW, CHARLES, HETHERINGTON, and RAISTRICK, *loc. cit.*
[2] COYNE, F. P., and H. RAISTRICK, *Biochem. Jour.*, **25**: 1513 (1931).
[3] RAISTRICK, H., and G. SMITH, *Biochem. Jour.*, **27**; 1814 (1933).

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CHAPTER XXXII

MOLD ENZYME PREPARATIONS: USES AND PRODUCTS

Reference has been made in Chap. XXIV to the types of enzymes that are elaborated by molds, while the use of living molds and mold preparations to saccharify starch in the manufacture of industrial alcohol has been discussed in Chap. III. Several enzyme preparations will now be considered in greater detail.

Commercial Amylase.—Commercial amylase or diastase is marketed under a variety of trade names, for example, Taka-diastase, Kashiwagi-diastase, Digestin, Polyzyme, Protozyme, and Oryzyme. Such preparations are generally mixtures of various enzymes. Purified enzyme preparations may be secured, however.[1]

Preparation.—The mold that is to be used for the enzyme preparation may be grown in trays in shallow layers or in rotating drums.

In the former method, which is the older one, bran (wheat or rice) is moistened thoroughly, steamed for 1 to 2 hr. to make soluble the starch and to destroy the undesirable microorganisms present, and then cooled to 25 to 30°C.[2] This cooled material is inoculated with the spores of a selected strain of *Aspergillus oryzae*. The inoculated and well-mixed mass is spread in trays, preferably with false bottoms, or on a suitable base, to a depth of approximately 1.5 in.[3] The temperature is maintained close to the optimum for the growth of the mold, usually about 30°C. A humidity sufficiently high to prevent drying of the bran and ventilation adequate to supply sufficient oxygen and to carry away some of the carbon dioxide are essential. The mold develops rapidly, and in 40 to 48 hr. a maximum of desirable enzymes are available in the mold mass. The product may be dried to prevent bacterial action; or the mass may be extracted at once with water (1 to 2 volumes for each volume of product), filtered or strained, and preserved in a manner suitable for the purpose for which the preparation is to be used.

If the extract is to be used for food or for medicinal purposes, sufficient ethanol is added to produce 70 per cent saturation. The enzymes are precipitated by the alcohol. The precipitate is washed, and thus

ANO, T., *Jour. Soc. Chem. Ind. (Japan)*, **40**: 37 (1937).

[2] WAKSMAN, S. A., and W. C. DAVISON, "Enzymes," The Williams & Wilkins Company, Baltimore, 1926.

[3] TAKAMINE, J., *Jour. Ind. Eng. Chem.*, **6**: 824 (1914).

dehydrated, with strong ethanol, dried and finally powdered. The resultant powder is whitish to whitish-yellow in appearance.

An alternate method is to concentrate the extract to a heavy sirup, using vacuum and a temperature of 30 to 40°C.

In case the enzyme preparation is not to be used as a food, the aqueous extract may be preserved by the use of a chemical antiseptic. The addition of sodium chloride to a concentration of about the saturation point (20 per cent) has been recommended.[1] Thymol, tricresols, phenol, and other substances have been used as preservatives.

According to Harada,[2] the essential factors in the preparation of enzymes are (1) the quality of the bran; (2) the moisture content of the bran; (3) the pH, the temperature, and the time of incubation; (4) the humidity; and (5) the use of sterilization. The selection of an appropriate mold is, of course, of prime importance[3] as not all strains of the *Aspergillus flavus-oryzae* group are equally potent in enzyme production.

The production of amylase preparations by the use of rotating drums with controlled aeration and temperature has been described by Underkofler, Fulmer, and Schoene[4] (see Chap. III). Products prepared by this method were high in potency.

Koji.—*Koji* is the Japanese name given to starters, which are used in several fermentations, among which are the sake (rice wine), *shoyu* (soy sauce), *miso* (soy cheese), and *shocho* (distilled alcoholic liquor) fermentations. *Koji* may be named according to the purpose for which it is used, for example, sake *koji* or *shoyu koji*. Its importance lies in its ability to digest starches and proteins with the production of soluble products, which may subsequently be acted upon by yeasts or bacteria.

The use of *koji* in the United States was introduced by Takamine.

Preparation of Koji.—*Koji* may be prepared in a number of ways and from various substances. Generally a low-priced, bulky, fibrous material, such as the bran of rice or wheat, is used. The substance to be used is sterilized, cooled, and inoculated with the spores of a selected strain of the *A. flavus-oryzae* group. The inoculated material is incubated at a temperature favorable to the growth of the mold until the enzymic activity is at a maximum. Unless the *koji* is used immediately, the mold growth should then be stopped by drying.

In one method of making *koji*,[5] whole brown rice is used. The rice is cleaned, soaked for a few hours in water, and then steamed. The

[1] Harada, T., *Ind. Eng. Chem.*, **23**: 1424 (1931).

[2] *Ibid.*

[3] Oshima, K., and M. B. Church, *Jour. Ind. Eng. Chem.*, **15**: 67 (1923).

[4] Underkofler, L. A., E. I. Fulmer, and L. Schoene, *Ind. Eng. Chem.*, **31**: 734 (1939).

[5] Morikawa, K., "A New Butyl and Isopropyl Alcohol Fermentation," doctor's dissertation in biology, Massachusetts Institute of Technology, Cambridge, 1926.

grains are thus swelled and cracked or burst. The steamed rice is spread out in trays in a layer approximately ½ in. thick to cool. Spores of *A. oryzae* are mixed thoroughly with the rice, which may then be heaped and covered with damp cloths to favor the growth of mold mycelium. After incubation at 25 to 30°C. for a few hours, during which the hyphae appear, the rice is again spread out in trays to a depth of about ½ in. and incubated until the period of maximum enzyme production.

During incubation of the rice in the trays, it is necessary to regulate the humidity carefully, for an excess of moisture may lead to bacterial spoilage of the molded rice, while insufficient moisture may not permit proper development of the molds.

Some Mold Products.—Molds have been used for many centuries in such countries as Japan and China on account of the desirable changes which they produce in various substances. In the preparation of soybean sauces, sake, and various other products, molds, especially of the *A. flavus-oryzae* group, assume much importance.

Soy sauce is a product manufactured from the soybean using *shoyu koji*. The strain of *A. flavus-oryzae* used should possess high proteolytic action, as well as amyloclastic, since there is very little starch in the beans used for the fermentations. The *koji* or starter is prepared by inoculating cooked soybeans, usually mixed with ground roasted wheat, with a selected strain of *A. flavus-oryzae* and incubating until each bean is covered with sporulating mycelium. The *koji* is placed in a concentrated sodium chloride brine wherein an enzymic digestion takes place over a long period of time—from a few months to a few years. Daily agitation and aeration are advantageous. Yeasts and bacteria aid in bringing about the changes in the bean. The mash which results is dark brown in color and of thick consistency. The mash is finally pressed, and the juice or sauce is boiled, filtered, and processed. Soy sauce is used in the manufacture of table sauces or it may be employed directly.

Tamari is a sauce prepared from soybeans, often with the addition of other materials such as rice. The flavor of *tamari* sauce differs from that of soy sauce. This difference in flavor is due to the use of *A. tamarii*, this mold being the dominant microorganism in this process. The fermentation period is shorter than that of the soy-sauce fermentation.

Miso is the name given to products prepared from cooked soy beans to which an *Aspergillus* starter and salt have been added and ripening permitted. There are several different types of *miso*. The concentration of the salt used, the flavoring in the ingredients employed, and the activity of the mold affect the type of final product. *Miso* is used especially as a breakfast food for children.[1]

[1] Ramsbottom, J., *Brit. Assoc. Advancement Sci., Annual Report,* 1936.

The Uses of Mold Enzymes.—Four principal types of mold enzymes may be used industrially: amylases (diastases), invertase, proteases, and pectinase.[1] Amylases, of which there are at least two kinds, alpha-amylase (dextrinogenic) and beta-amylase (saccharogenic), and which may be produced from either molds or bacteria, are used in the preparation of sizes and adhesives, in the desizing of textiles, for the removal of starch from apple pomace in the manufacture of pectin, in the pharmaceutical trade, and for other purposes. In each of these cases the action of the amylases is initially upon starch, and, after the desired conversion of this substance has taken place, the enzymes may be destroyed by the application of heat.

Invertase, which may be produced from yeasts or molds, is used in the confectionery industry for the making of soft centers in chocolate-coated candies, for this enzyme converts sucrose to a mixture of glucose and fructose. This enzyme may also be used in the making of non-crystallizable sirups from sucrose, by a partial hydrolysis of this sugar.

Proteases, which may be obtained from *A. flavus*, or from bacteria, are used also for several purposes. They may be used in the degumming of silk goods, in the unhairing and bating of hides, in the manufacture of liquid glue, as a substitute for or combined with soap in the laundry business, and as an agent in the ripening of cheese. In the preparation of liquid glue, proteases partially hydrolyze the glue. Proteases are said to be the best agent used for this purpose in the preparation of liquid glue,[2] as controlled operations may thus be carried out. Proteases may also be used in making chillproof beer.

The term "proteases," as used, refers to a mixture of proteolytic enzymes, which may include true proteinases, and peptidases or ereptases (polypeptidases, dipeptidases).[3]

Pectinase,[2] usually from penicillia,[3] may be used to aid in the clarification of fruit juices. Enzymes that hydrolyze pectin are also important in retting processes, as in the manufacture of linen from flax.

An enzyme "kinase" was found by Kunitz,[4] which had the ability to convert trypsinogen to trypsin in an acid medium. This enzyme was produced by a species of *Penicillium*.

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[1] Willaman, J. J., Abstracts of Communications, Third International Congress of Microbiology, New York, p. 335, Sept. 2–9, 1939.
[2] *Ibid.*
[3] Wallerstein, L., *Ind. Eng. Chem.*, **31**: 1218 (1939).
[4] Kunitz, M., *Jour. Gen. Physiol.*, **21**: 601 (1938).

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-: Process of Chillproofing and Stabilizing Beers and Ales, U.S. Patents 2,077,-447, 2,077,448, Apr. 20, 1937.

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CHAPTER XXXIII

THE PRODUCTION OF FAT BY MOLDS

Historical.—In 1906, Browne[1] reported the results of some analyses made on the dried mycelium of a species of *Citromyces* (*Penicillium*) that had grown as a scum on the surface of tanks of leftover molasses in a hot room of a sugar factory. The mycelium contained 27.50 per cent of fat, which in several physical and chemical ways resembled butterfat, for example, in respect to the saponification number, the Reichert-Meissl number, the melting point, and the iodine number. The fat differed, however, from butterfat in other respects, for example, in the acid number, and the mean molecular weight of the soluble and the mean molecular weight of the insoluble acids. The fat contained a preponderance of caproic [CH_3 $(CH_2)_4 \cdot COOH$] and caprylic [$CH_3(CH_2)_6 \cdot COOH$] acids.

Oleic, palmitic, and two unidentified acids were detected by Sullivan[2] in the alcoholic soda extract of the dried mycelium of *Penicillium glaucum*, that had been grown on Raulin's medium.

Belin[3] (1926) mentioned fat production by the genus *Aspergillus*.

Rockwell and O'Flaherty[4] (1931), during a study of the physiology of some of the common molds, analyzed the mycelia of some of them. They discovered 0.58 per cent of fat (by ether extraction) in the moist mycelium of *Aspergillus niger*.

Barber[5] investigated fat production by a green species of *Penicillium*. The species appeared to grow equally well on nutrient solutions of sucrose, glucose, or xylose, but not as well on glycerol. In each case, the same fat or mixture of fats was apparently produced. The ether extract of the mycelia contained palmitic, stearic, oleic, alpha-linoleic, and beta-linoleic acids, free and as glycerides. Sterols were also found. A yield of 14 per cent fat was obtained from the dried mycelium of the mold when grown on a 5 per cent sucrose solution.

The energy relations involved during the production of fat from sugar by *A. niger* were studied by Terroine and Bonnet[6] (1927).

[1] Browne, C. A., Jr., *Jour. Am. Chem. Soc.*, **28**: 465 (1906).

[2] Sullivan, M. X., *Science*, **38**: 678 (1913).

[3] Belin, P., *Bull. soc. chim. biol.*, **8**: 1081 (1926).

[4] Rockwell, G. E., and F. O'Flaherty, *Jour. Am. Leather Chem. Assoc.*, **26**: 216 (1931).

[5] Barber, H., *Jour. Soc. Chem. Ind.* (*Trans.*), **46**: 200T (1927); *Biochem. Jour.*, **33**: 1158 (1929).

[6] Terroine, E. F., and R. Bonnet, *Bull. soc. chim. biol.*, **9**: 588 (1927).

The relation of the temperature at which the molds were grown to the degree of unsaturation of the fatty acids produced was studied by Pearson and Raper.[1] Using the iodine number as a measure of the degree of unsaturation, they showed that both *A. niger* and *Rhizopus nigricans* produced more unsaturated acids at low temperatures than at higher temperatures.

Pontillon[2] studied the effect of the constituents of the medium, inorganic and organic, on the quality and quantity of fat produced by *A. niger*.

THE NATURE OF THE FATS PRODUCED BY MOLDS

Lipids of Penicillium javanicum van Beijma.—The mold was cultivated on a 20 per cent nutrient glucose solution at 30°C., and the dried mycelium was extracted with a redistilled petroleum ether, yielding 11 per cent of oil.[3] Physical and chemical characteristics of the oil are shown in the accompanying table.

TABLE 81.—PHYSICAL AND CHEMICAL CHARACTERISTICS OF OIL FROM *P. javanicum*[1]

Solidification point, °C.	6–7
Melting point, °C. about	15
Specific gravity (25°/25°)	0.9145
Refractive index (25°)	1.4680
Acid value	10.6
Saponification value	191
Iodine value (Hanus)	84.0
Reichert-Meissl value	0.3
Acetyl value	10.7
Unsaponifiable matter, per cent	2.00
Saturated acids (corrected), per cent	30.8
Unsaturated acids (corrected), per cent	60.8
Melting points of mixed saturated acids, °C.	52.5
Mean molecular weight of saturated acids	272

[1] WARD, G. E., and G. S. JAMIESON, *Jour. Am. Chem. Soc.*, **56**: 973 (1934).

Analyses of the unsaturated and saturated fractions follow:

TABLE 82.—ANALYSIS OF UNSATURATED FRACTION[1]

	In unsaturated fraction, per cent	In oil	
		Acid, per cent	Glyceride, per cent
Oleic acid	52.1	31.7	33.2
Linoleic acid	47.9	29.1	30.5
Total	100.0	60.8	63.7

[1] WARD, G. E., and G. S. JAMIESON, *Jour. Am. Chem. Soc.*, **56**: 973 (1934).

[1] PEARSON, L. K., and H. S. RAPER, *Biochem. Jour.*, **21**: 875 (1927).
[2] PONTILLON, C., *Rev. gén. botan.*, **44**: 465, 526 (1932); **45**: 20 (1933).
[3] WARD, G. E., and G. S. JAMIESON, *Jour. Am. Chem. Soc.*, **56**: 973 (1934).

TABLE 83.—ANALYSIS OF THE SATURATED FRACTION

	In saturated fraction, per cent	In oil	
		Acid, per cent	Glyceride, per cent
Palmitic acid	69.5	21.4	22.4
Stearic acid	28.0	8.6	9.0
Tetracosanic acid	2.5	0.8	0.8
Total	100.0	30.8	32.2

Lipids of Aspergillus sydowi.—The alcohol-ether extract of the dried mycelium of *A. sydowi* contained oleic, linoleic, palmitic, stearic, and *n*-tetracosanic acids; glycerol; and sterols as shown in the following

TABLE 84.—COMPOSITION OF THE SIMPLE LIPIDS OF *A. sydowi*[1,2]

Fatty acids	80.8
Volatile acids (calculated as butyric)	0.46
Saturated acids	22.6
Palmitic	8.8
Stearic	11.0
N-Tetracosanic	0.9
Unsaturated acids	52.9
Oleic	29.6
Linoleic	16.3
Higher acids	1.7
Unsaponifiable	8.18
Total sterols[3]	5.36
Glycerol	4.2

[1] STRONG, F. M., and W. H. PETERSON, *Jour. Am. Chem. Soc.*, **56**: 952 (1934).
[2] Figures indicate percentage of the original lipids.
[3] Based on the colorimetric sterol determination.

Lipids of Penicillium aurantio-brunneum.[1]—The mold was grown on a glucose-inorganic salt solution in the manner described by Peterson

TABLE 85.—APPROXIMATE COMPOSITION OF THE SIMPLE LIPIDS

	Per Cent
Total fatty acids	85.4
Oleic[1]	40.2
Linoleic[1]	31.2
Palmitic[2]	8.6
Stearic[2]	5.3
Unsaponifiable	4.5
Ergosterol	1.9
Glycerol	3.1

[1] Calculated from the weight and iodine number of the unsaturated acids.
[2] Calculated from the weight and neutral equivalent of the crude saturated acids.

[1] KROEKER, E. H., F. M. STRONG, and W. H. PETERSON, *Jour. Am. Chem. Soc.*, **57**: 354 (1935).

TABLE 86.—COMPOSITION OF CERTAIN MOLDS[1]

Organism	Glucose-inorganic-salts medium						Glucose-malt-sprouts medium					
	Weight of dry pad, grams per 100 cc.	Sterol, per cent	Lipid, per cent	Crude protein (N × 6.25), per cent	Carbohydrate (by difference),[2] per cent	Free fatty acid of lipid (as oleic), per cent	Weight of dry pad, grams per 100 cc.	Sterol, per cent	Lipid, per cent	Crude protein (N × 6.25), per cent	Carbohydrate (by difference),[3] per cent	Free fatty acid of lipid (as oleic), per cent
Aspergillus aerea 4700A	2.67	0.65	4.7	34.4	60.9	....	2.55	0.88	6.1	25.6	68.3	
Aspergillus carbonarius 4030.1	4.04	0.39	1.1	13.7	85.2	56.3	5.03	0.33	2.1	12.5	85.4	
Aspergillus cinnamomeus 3534 B.	3.63	0.42	3.4	25.0	71.6	....	4.16	0.54	5.0	14.4	80.6	
Aspergillus citrosporus 4301.10	3.53	0.45	3.9	32.5	63.6	....	2.59	0.57	1.5	25.6	72.9	
Aspergillus clavatus 107	2.03	0.75	7.6	35.0	57.4	20.0	2.77	0.86	16.6	22.5	60.9	
Aspergillus fischeri 5041	2.62	0.90	6.4	31.2	62.4	15.2	2.40	0.84	13.4	28.1	58.5	8.3
Aspergillus flavipes 2	2.81	0.75	7.0	36.3	57.7	8.0	2.53	0.77	23.0	21.2	55.8	
Aspergillus fumigatus 2	3.13	0.82	3.1	31.2	65.7	51.5	2.63	0.26	1.8	24.4	73.8	73.1
Aspergillus fuscus 3534c	2.81	0.93	3.0	28.1	68.9	30.2	3.94	0.48	5.7	18.1	76.2	16.9
Aspergillus insuetus 4658.245	2.52	1.10	13.5	23.7	62.8	13.4	2.32	1.05	24.4	21.9	53.7	15.0
Aspergillus lutea 4640.473	2.62	0.65	3.8	36.7	59.5	....	2.41	0.45	4.8	26.2	69.0	
Aspergillus melleus 4291.6	1.98	0.88	3.1	40.6	56.3	44.9	2.55	0.95	3.8	23.7	72.5	36.2
Aspergillus minutus 4894.2	2.33	0.52	7.4	32.5	60.1	27.4	2.56	1.10	18.3	23.7	58.0	10.6
Aspergillus nidulans 1	3.45	0.55	19.9	25.6	54.5	9.7	4.16	0.38	16.8	13.1	70.1	7.9
Aspergillus niger 2	3.14	0.80	2.6	28.1	69.3	....	3.87	0.43	2.8	18.1	79.1	
Aspergillus ochraceous 4700A	2.21	0.61	4.5	43.1	52.4	19.7	2.98	0.44	5.2	21.9	72.9	24.3
Aspergillus oryzae 965	1.17	0.84	5.0	33.7	61.3	19.5	1.45	1.09	5.6	36.3	58.1	19.9
Aspergillus schiemanni 3534c	2.39	0.78	4.1	32.5	63.4	34.2	3.47	0.81	5.2	18.1	76.7	38.5
Aspergillus sydowi 1	2.02	1.09	5.0	26.9	68.1	8.4	2.53	0.86	5.2	20.6	74.2	8.0
Penicillium aurantio-brunneum 4733.5	1.72	0.87	11.8	23.7	64.5	17.6	2.21	0.77	12.9	20.6	66.5	10.7
Penicillium chrysogenum 4733.33	1.66	0.91	2.3	43.7	54.0	67.8	1.87	1.16	7.1	15.0	77.9	30.4
Penicillium cyaneum 4640.422	0.75	0.59	2.4	33.1	64.5	....	1.82	0.70	4.4	20.6	75.0	
Penicillium cyaneo-fulvum 4733.47	1.06	0.40	3.2	36.3	60.5	39.4	1.71	0.38	8.2	21.9	69.9	41.2
Paecilomyces varioti 1	1.64	1.25	14.9	30.0	55.1	14.8	1.48	1.70	10.1	26.9	63.0	27.4
Average	2.41	0.75	6.0	31.6	62.5	27.6	2.92	0.78	8.8	22.5	69.6	26.2

[1] PRUESS, L. M., E. C. EICHINGER, and W. H. PETERSON, *Centr. Bakt. Parasitenk.* Abt. II, **89**: 370 (1934).
[2] Includes ash; this varies from 2.5 to 8.5 per cent.
[3] Includes ash; this varies from 1.0 to 4.5 per cent.

and his associates. The mycelial mats[1] were steamed to destroy the mold, dried at 65°C., ground finely, and extracted with a 1:1 mixture of alcohol and ether. The dried mycelium yielded 11.6 per cent crude lipids.

Table 85 shows the approximate composition of the simple lipids, which were mainly the glycerides of palmitic, stearic, oleic, and linoleic acids.

The Lipid Content of Molds.—The lipid content of different molds varies considerably. On the basis of the dry weight, the mycelium may contain as much as 41.5[2] or as little as 1 per cent of lipid. Mold spores have been reported to contain from 1 to 14 per cent of lipid.[3]

Pruess, Eichinger, and Peterson cultivated 24 molds on 2 different types of media: (1) a glucose-inorganic salts medium containing calcium carbonate in excess and (2) a glucose–malt-sprouts medium. Results of the analyses of the dried mycelia of the molds are shown in Table 86. The lipid content of the mycelia varied from 1.1 to 19.9 per cent, with an average of 6.0 per cent, when the molds were grown on the glucose-inorganic salts (synthetic) medium; and from 1.5 to 24.4 per cent, with an average of 8.8 per cent, when the molds were grown on the glucose–malt-sprouts (organic) medium. The average lipid content of all the molds was over 46 per cent greater when grown on the organic medium than when grown on the synthetic medium. However, some molds, for example, *Aspergillus nidulans* 1 and *Paecilomyces variota* 1, yielded more lipid on the latter medium than on the former medium.

Ward and his associates extracted the crude fat from 61 different molds, 39 penicillia and 22 aspergilli, using ethyl ether. Of these molds, 10 contained more than 15 per cent, but only 6 more than 20 per cent of crude fat, as indicated in Table 87.

TABLE 87.—SOME MOLDS CONTAINING MORE THAN 15 PER CENT OF CRUDE FAT[1]

Mold	Crude Fat in Dried Mycelium. Per Cent
Penicillium bialowiezense	17.0
P. citrinum Thom	18.1
P. hirsutum Dierckx	18.4
P. soppi Zal	20.2
P. javanicum van Beijma	22.2
P. roqueforti Thom	22.9
P. oxalicum Currie and Thom	24.4
P. piscarum Westling	26–28
P. flavocinerium Biourge	28.5
Aspergillus flavus Thom and Church	16.0

[1] WARD, LOCKWOOD, MAY, and HERRICK, *loc. cit.*

[1] PETERSON, W. H., L. M. PRUESS, H. J. GORCIA, and H. C. GREENE, *Ind. Eng. Chem.*, **25**: 213 (1933).

[2] WARD, G. E., L. B. LOCKWOOD, O. E. MAY, and H. T. HERRICK, *Ind. Eng. Chem.*, **27**: 318 (1935).

[3] PRUESS, L. M., E. C. EICHINGER, and W. H. PETERSON, *Centr. Bakt. Parasitenk.*, Abt. II, **89**: 370 (1934).

FACTORS AFFECTING THE LIPID CONTENT OF MOLDS

Sufficient data have already been presented to indicate the importance of the species of mold in respect to the lipid content. Cultural conditions are likewise important: the concentration of the sugar, the kind and quantity of nitrogen-containing substance, the presence of small quantities of materials that stimulate fat production, the pH, the temperature, and the incubation period. The effect of cultural conditions on two molds: *Penicillium javanicum* van Beijma and *Aspergillus fischeri* are discussed in the succeeding paragraphs.

Fat Production by Penicillium javanicum van Beijma.[1]—This mold was selected for study since it produced the heaviest mats, yielding a considerable amount of lipid. The mold was described by Beijma.[2]

The mold was cultivated in 200-cc. Pyrex Erlenmeyer flasks, into each of which were placed 75 cc. of the following medium:

	Grams per Liter
Glucose	200
NH_4NO_3	2.25
KH_2PO_4	0.3
$MgSO_4 \cdot 7H_2O$	0.25

A chemically pure glucose (anhydrous) was used when the effect of the ions of various salts was ascertained, otherwise a commercial grade of glucose containing 91.5 per cent of pure glucose, 8 per cent water, and 0.4 per cent dextrin was used.

The pH of the medium was 4 to 5; the temperature of incubation, 30°C.; and the period of incubation 12 days.

In the following table is shown the effect of varying the glucose concentration:

TABLE 88.—THE EFFECT, ON FAT PRODUCTION, OF VARYING THE GLUCOSE CONCENTRATION[1]

Glucose, per cent	Mat weight, per cent	Fat, per cent	Culture acid, equivalent cc. N/10 acid	Glucose consumed, grams
20	2.522	29.0	149	10.3
30	2.400	34.6	209	11.3
40	1.964	41.5	148	9.9
50	1.021	35.2	20	5.2

[1] LOCKWOOD, L. B., G. E. WARD, O. E. MAY, H. T. HERRICK, and H. T. O'NEILL, *Centr. Bakt. Parasitenk.*, Abt. II, **90**: 411 (1934).

[1] LOCKWOOD, L. B., G. E. WARD, O. E. MAY, H. T. HERRICK, and H. T. O'NEILL, *Centr. Bakt. Parasitenk.*, Abt. II, **90**: 411 (1934).

[2] BEIJMA THOE KINGMA, F. H. VAN, *Verhandel. Akad. Wetensch. Amsterdam, Afdeel. Naturkunde*, **26** (4): 16 (1929).

The greatest mat weight occurred when the glucose concentration was 20 per cent; the greatest titrable acidity at 30 per cent; and the greatest percentage of fat at 40 per cent.

Xylose, galactose, maltose, sucrose, glycerol, starch, and dextrin yield lipids, xylose being a particularly good source of carbon.

P. javanicum utilized sodium, potassium, magnesium, or calcium nitrates; sodium nitrite; ammonium chloride or ammonium sulphate as sources of nitrogen; but ammonium nitrate was the best source. Ammonium chloride and ammonium sulphate caused the pH to drop too low.

The growth and metabolism of *P. javanicum* were favored by the presence of 2.25 to 3.375 g. of NH_4NO_3, 0.3 to 1.2 g. of KH_2PO_4, and about 0.25 g. of $MgSO_4 \cdot 7H_2O$ per liter of medium.

Chromic, columbic, ferric, molybdic, and tungstic ions stimulated mycelial growth, fat and citric acid formation, and glucose consumption.

The pH range of 3.1 to 6.8 was favorable, the final pH of the medium being about 2.5 in each case.

Increasing the air pressure within the culture flask tended to inhibit the metabolism of *P. javanicum.*

Decreasing the ratio of surface area to volume decreased the efficiency of the conversion of glucose to lipids.

The production of fat from glucose by *P. javanicum* in a large-scale laboratory apparatus is described by Ward, Lockwood, May, and Herrick in a later report.[1]

The composition of two media used for growing molds for lipid production, known as "*G* solution" and "*M* solution," of which *M* solution gave the more consistent results, follows:[1]

TABLE 89.—SOME MEDIA USED FOR GROWING MOLDS FOR LIPID PRODUCTION

G solution		*M* solution	
Substance	Grams per liter	Substance	Grams per liter
Commercial glucose	220	Commercial glucose	220
NH_4NO_3	4.50	NH_4NO_3	2.25
KCl	0.40	KH_2PO_4	0.3
H_3PO_4	0.216	$MgSO_4 \cdot 7H_2O$	0.25
$MgSO_4 \cdot 7H_2O$	2.00		

Fat Production by Aspergillus fischeri.—Prill, Wenck, and Peterson have studied the effect of various factors, such as glucose concentration, ammonium nitrate concentration, acidity, alkalinity, temperature, aeration, and incubation period, on fat production by *A. fischeri.*[2]

[1] WARD, LOCKWOOD, MAY, and HERRICK, *loc. cit.*

[2] PRILL, E. A., P. R. WENCK, and W. H. PETERSON, *Biochem. Jour.*, **29**: 21 (1935).

The mold was cultured at 30°C. in 500-cc. Pyrex Erlenmeyer flasks, each of which contained 100 cc. of the following solution, except where the nature of the experiment required otherwise.

	Grams
Commercial glucose (cerelose)	20
NH_4NO_3	1.00
KH_2PO_4	0.68
$MgSO_4 \cdot 7H_2O$	0.50
$FeCl_3 \cdot 6H_2O$	0.016
$ZnSO_4 \cdot 7H_2O$	0.005
Distilled water, to make 100.0 cc.	
$CaCO_3$	

The mold growth was destroyed at the end of the incubation period by autoclaving it at 120°C. for 10 min. Mycelial mats were then washed with water, dried for 2 days at 37°C., ground, and extracted with hot absolute alcohol for 12 hr. or more.

A high fat content[1] in the mycelium was favored by a high concentration of glucose, a low concentration of ammonium nitrate (NH_4NO_3), and a neutral or slightly alkaline solution. (Compare with fat production by *Endomyces vernalis*.) For further details consult the paper by Prill, Wenck, and Peterson.[1]

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[1] *Ibid.*

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WOOLLEY, D. W., F. M. STRONG, W. H. PETERSON, and E. A. PRILL: The Chemistry of Mold Tissue. X. The Phospholipides of *Aspergillus Sydowi, Jour. Am. Chem. Soc.*, **57**: 2589 (1935).

CHAPTER XXXIV

SOME MINOR CHEMICAL ACTIVITIES OF THE LOWER FUNGI

One of the most interesting aspects of microbiology is the biochemistry molds. Because of their ubiquity and the mischief these organisms, once regarded as biological outcasts, can do in many branches of industry, their study has distinct economic importance. This study of the lower fungi has advanced rapidly since about 1922. The outstanding work of Prof. Raistrick and his associates is mentioned in particular, because of the new and careful methods of investigation they have devised. A large number of papers, many of which are cited at the end of this chapter, have been published on various aspects of this organized research.

A study of Table 68 (page 349) will give the reader some idea as to the diversified metabolic products formed by the lower fungi. Methods for production of some of the most important substances mentioned in this table have been discussed in some detail in the preceding chapters. The scope of this text does not permit extended consideration of others, but the interested reader will find excellent reviews of papers by Birkinshaw,[1] Clutterbuck,[2] Iwanoff and Zwetkoff,[3] Lockwood and Moyer,[4] Raistrick,[5] and others. Likewise at the end of this chapter there will be found a large number of references to the literature.

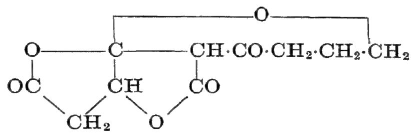
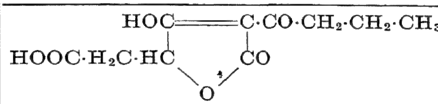
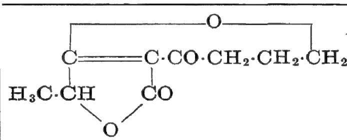
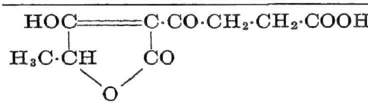
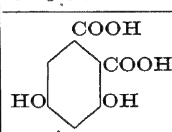
ACIDS

A large number of organic acids are produced by molds although at the present time only a few of them are of commercial importance. These have already been discussed. Brief mention will now be made of some other organic acids that are metabolic products of molds.

Succinic acid is produced by several species of *Mucor*, by a white species of *Aspergillus;* by *A. terreus*, *Penicillium aurantio-virens*, and *Fumago vagans;* and by other molds. Succinic acid may originate from cell proteins or from the fermentation of carbohydrates. Its exact origin is not entirely clear.

[1] Birkinshaw, J. H., *Biol. Rev.*, **12**: 357–391 (1937).
[2] Clutterbuck, P. W., *Jour. Soc. Chem. Ind.*, **55**: 55T-61T (1936).
[3] Iwanoff, N. N., and E. S. Zwetkoff: *Ann. Rev. Biochem.*, **5**: 585–612 (1936).
[4] Lockwood, L. B., and A. J. Moyer, *Bot. Rev.*, **4**: 140–164 (1938).
[5] Raistrick, H., *Ergeb. Enzymforsch.*, **1**: 345 (1932); **7**: 316–349 (1938).

TABLE 90.—SOME ACIDS FORMED BY MOLDS

Acid	Formula	Produced by
Aconitic[69]* ($C_6H_6O_6$)	$CH_2\cdot COOH$ | $C\cdot COOH$ ‖ $CH\cdot COOH$	*Aspergillus itaconicus*
Byssochlamic[35] ($C_{18}H_{20}O_6$)		*Byssochlamys fulva*
Carlic (anhydrous)[36,43] ($C_{10}H_{10}O_6$)		*Penicillium charlesii* G. Smith
Carlosic[36,43] ($C_{10}H_{12}O_6$)		*P. charlesii* G. Smith
Carolic (anhydrous)[36,41] ($C_9H_{10}O_4$)		*P. charlesii* G. Smith
Carolinic[36,41] ($C_9H_{10}O_6\cdot H_2O$)		*P. charlesii* G. Smith
Citric ($C_6H_8O_7$)	$CH_2\cdot COOH$ | $C(OH)\cdot COOH$ | $CH_2\cdot COOH$	*A. niger*, etc. See Chap. XXV
3:5-Dihydroxy-phthalic acid[25] ($C_8H_6O_6$)		Some species and strains of the *P. brevi-compactum* series
Dimethyl pyruvic[69] ($C_5H_8O_3$)	CH_3 $CH_3—\underset{H}{C}\cdot CO\cdot COOH$	*A. niger*

* The numbers in parentheses refer to the citations at the end of the chapter.

TABLE 90.—SOME ACIDS FORMED BY MOLDS.—(*Continued*)

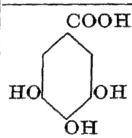
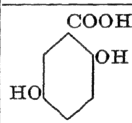
Acid	Formula	Produced by
Fulvic[44] ($C_{14}H_{12}O_8$)		*P. griseo-fulvum* Dierckx *P. flexuosum* Dale *P. brefeldianum* Dodge
Fumaric ($C_4H_4O_4$)	CH·COOH ‖ CH·COOH	Species of the genera *Rhizopus*, *Mucor*, etc.
Gallic (3:4:5-trihydroxybenzoic) ($C_7H_6O_5$)		*A. niger* See Chap. XXVIII
Gentisic[29] (2:5-dihydroxybenzoic) ($C_7H_6O_4$)		*P. griseo-fulvum* Dierckx
Glaucic[92] ($C_{17}H_{22}O_5$)		*A. glaucus*
Glauconic[69] I ($C_{18}H_{20}O_7$) II ($C_{18}H_{20}O_6$)		Some green *Penicillium* species
d-Gluconic ($C_6H_{12}O_7$)	COOH | HCOH | HOCH | HCOH | HCOH | CH_2OH	*A. niger* *P. chrysogenum*, etc. See Chapter XXVI
Glucuronic[98] ($C_6H_{10}O_7$)	CHO | $(CHOH)_4$ | COOH	*Ustulina vulgaris*
Glycolic ($C_2H_4O_3$)	CH_2OH | COOH	*A. niger*
Glyoxylic ($C_2H_2O_3$)	CHO | COOH	*A. niger*

TABLE 90.—SOME ACIDS FORMED BY MOLDS.—(*Continued*)

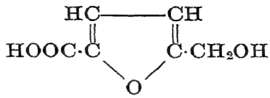
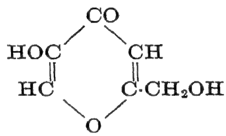
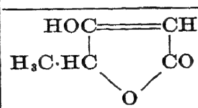
Acid	Formula	Produced by
2-Hydroxymethyl-furane-5-carboxylic[91] ($C_6H_6O_4$)		*A. glaucus* *A. clavatus* *A. niger* *A. oryzae* *A. wentii*
Itaconic[81,63] ($C_5H_6O_4$)	$H_2C{=}C{\cdot}COOH$ $\quad\vert$ $CH_2{\cdot}COOH$	*A. itaconicus*
Kojic ($C_6H_6O_4$)		*A. flavus*, etc. See Chap. XXIX
γ-Ketopentadecoic[91] ($C_{15}H_{28}O_2$)	CH_3 $(CH_2)_9$ CH_2 CO CH_2 $CH_2{\cdot}COOH$	*P. spiculisporum* Lehman
Lactic ($C_3H_6O_3$)	CH_3 $CHOH$ $COOH$	Species of the genera *Rhizopus* and *Mucor*, etc. See Chap. XXVII
Malic ($C_4H_6O_5$)	$CH_2{\cdot}COOH$ $CHOH{\cdot}COOH$	*A. fumaricus* *A. niger*, etc.
d-Mannonic ($C_6H_{12}O_7$)	$COOH$ $HOCH$ $HOCH$ $HCOH$ $HCOH$ CH_2OH	*P. purpurogenum* var. *rubrisclerotium*
l-γ-Methyltetronic[45] ($C_5H_6O_3$)		*P. charlesii* G. Smith

TABLE 90.—SOME ACIDS FORMED BY MOLDS.—(*Continued*)

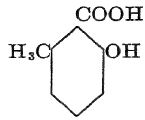
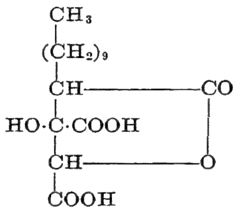
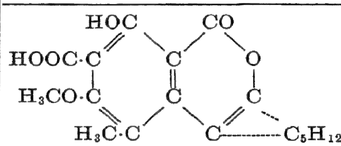
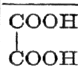
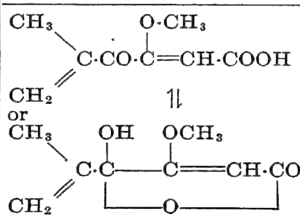
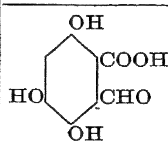
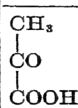
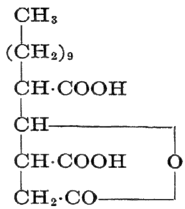
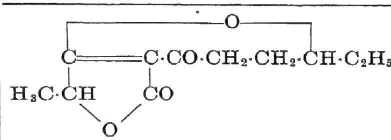
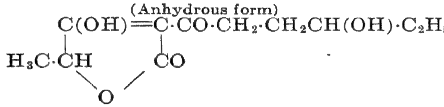
Acid	Formula	Produced by
6-Methylsalicylic[19,91] (2-Hydroxy-6-methylbenzoic) ($C_8H_8O_3$)		*P. griseo-fulvum* Dierckx *P. flexuosum* Dale
Minioluteic[38] ($C_{16}H_{26}O_7$)		*P. minio-luteum* Dierckx
Mycophenolic[31] ($C_{17}H_{20}O_6$)		Some species and strains of the *P. brevi-compactum* series
Oxalic ($C_2H_2O_4$)		*A. niger* etc.
Penicillic[48] ($C_8H_{10}O_4$)		*P. puberulum* Bainier *P. cyclopium* Westling
Puberulic[23] ($C_8H_6O_6$)		*P. aurantio-virens* *P. puberulum*
Pyruvic (pyro-racemic)[69] ($C_3H_4O_3$)		*A. niger*

TABLE 90.—SOME ACIDS FORMED BY MOLDS.—(*Continued*)

Acid	Formula	Produced by
Spiculisporic[16,38] ($C_{17}H_{28}O_6$)		*P. spiculisporum* Lehman *P. crateriforme* Gilman and Abbott *P. minio-luteum* Dierckx
Succinic ($C_4H_6O_4$)	$CH_2{\cdot}COOH$ | $CH_2{\cdot}COOH$	Species of the genera *Rhizopus, Mucor, Fusarium*, etc.
Terrestric[52] ($C_{11}H_{14}O_4$)	 (Anhydrous form)  (Hydrate form)	Strains of *P. terrestre* Jensen series

Malic acid has been reported as a product of *A. niger*, *A. fumaricus*, *A. flavus* Link, a white species of *Aspergillus*, and a species of *Clasterosporium*. It is produced in small amounts.

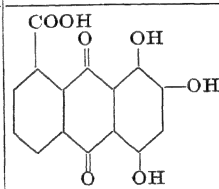
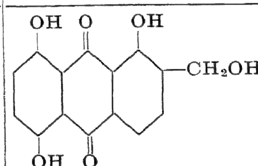
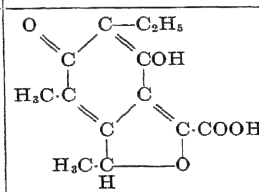
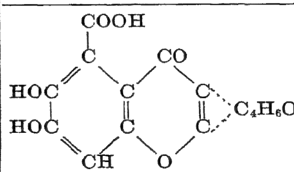
Itaconic acid is produced by *A. itaconicus* (Kinoshita) and by *A. terreus* Thom.[1] Yields of as high as 11.8 per cent of itaconic acid were produced from the glucose metabolized by *A. terreus* Thom. Five other strains of *A. terreus* failed to produce itaconic acid.

From the metabolic products of *P. charlesii* G. Smith several organic acids have been isolated. Carolic, carolinic, carlic, and carlosic acids (see Table 90) were first described by Clutterbuck and his associates.[2] The first two of these acids are dextrorotatory, while the last two are levorotatory. They may be obtained by growing the mold on the Czapek-Dox medium, but larger yields of carolinic acid are obtained on the Raulin-Thom medium, while no carlosic acid has been isolated from

[1] CALAM, C. T., A. E. OXFORD, and H. RAISTRICK, *Biochem. Jour.*, **33**: 1488 (1939).

[2] CLUTTERBUCK, P. W., W. N. HAWORTH, H. RAISTRICK, G. SMITH, and M. STACEY, *Biochem. Jour.*, **28**: 94–110 (1934).

TABLE 91.—SOME MOLD PIGMENTS

Pigment	Structural formula	Produced by
Aurantin (yellow) ($C_{16}H_{22}O_3$)		*Oōspora aurantia* (Cooke) Sacc. & Vogl.(69)*
Aurofusarin (orange-yellow) ($C_{30}H_{20}O_{12}$)		*Fusarium culmorum*(53) (W. G. Smith) Sacc.
Auroglaucin (orange) ($C_{19}H_{22}O_3$)		Species of *Aspergillus glaucus* series(40)
Beta-carotene (yellow)		*Mucor hiemalis*(69) *Phycomyces blakeseeanus*
Boletol (blue) ($C_{15}H_8O_7$)		*Boletus luridus*(82) *B. satanas* *B. strobilaceus*
Catenarin [β-(hydroxy-methyl)-1:5:8-trihy-droxyanthraquinone] (red) ($C_{15}H_{10}O_6$)		*Helminthosporium catenarium*(37) Drechsler *H. gramineum* Rabenhorst *H. tritici-vulgaris* Nisikado *H. velutinum*
Chrysogenin (yellow) ($C_{18}H_{22}O_6$)		*Penicillium chrysogenum*(26)
Citrinin (yellow) ($C_{13}H_{14}O_5$)		*P. citrinum* Thom(14) *A. terreus*
Citromycetin (yellow) ($C_{14}H_{10}O_7 \cdot 2H_2O$)		Species of *Citromyces*(11) *C. glaber* *C. pfefferianus*

* Numbers in parentheses refer to bibliography at the end of the chapter.

TABLE 91.—SOME MOLD PIGMENTS.—(*Continued*)

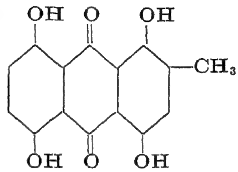
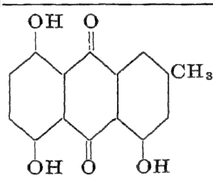
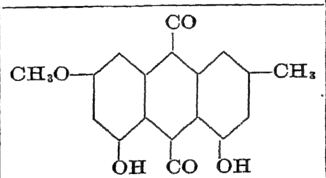
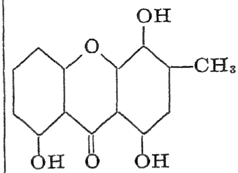
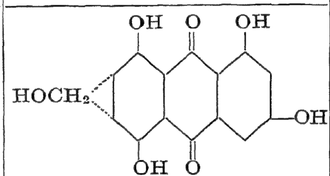
Pigment	Structural formula	Produced by
Cynodontin (probably 1:4:5:8-tetrahydroxy-2-methyl-anthraquinone) (bronze) ($C_{15}H_{10}O_6$)		*H. avenae* Eidam[32] *H. cynodontis* Marignoni *H. euchlaenae* Zimmermann
Erythroglaucin (dark red) ($C_{16}H_{12}O_6$)		*A. ruber*[40,62] Species of *A. glaucus* series
Flavoglaucin (lemon-yellow) ($C_{19}H_{28}O_3$)		Species of *A. glaucus* series[40]
Fulvic acid (yellow) ($C_{14}H_{12}O_8$)		*P. brefeldianum*[44] Dodge *P. flexuosum* Dale *P. griseo-fulvum* Dierckx
Helminthosporin (2-methyl-4:5:8-trihydroxyanthraquinone) (dark-maroon crystals) ($C_{15}H_{10}O_5$)		*H. gramineum* Rabenhorst[28] *H. catenarium* Drechsler *H. cynodontis* Marignoni *H. tritici-vulgaris* Nisikado
Monascoflavin (yellow) ($C_{17}H_{22}O_4$)		*Monascus purpureus* Went[69] (from old mycelium)
Monascorubrin (red) ($C_{22}H_{24}O_5$)		*Monascus purpureus* Went[69]
Ochracin		*A. ochraceus*[84]
Oösporin (purple-brown with $FeCl_3$) ($C_{10}H_{14}O_2$)		*Oöspora aurantia* (Cooke) Sacc. & Vogl.[69]
Phoenicin (red)		*P. phoeniceum*[77] (old cultures)
Physcion		Species of *A. glaucus*[62] series

TABLE 91.—SOME MOLD PIGMENTS.—(*Continued*)

Pigment	Structural formula	Produced by
Ravenelin (3-Methyl-1:4:8-trihydroxyxanthone) (yellow) ($C_{14}H_{10}O_5$)		*H. ravenelii* Curtis[50] *H. turcicum* Passerini
Rubrofusarin ($C_{15}H_{12}O_5$)		*F. culmorum* (W. G. Smith) Sacc.[53]
Tritisporin [1:3:5:8-Tetrahydroxy-6-(or 7-)-(hydroxymethyl)-anthraquinone] (reddish brown) ($C_{15}H_{10}O_7$)		*H. tritici-vulgaris* Nisikado[37]

the latter medium. Carolic and carolinic acids[1] are derivatives of *l*-γ-methyltetronic acid, and are structurally closely related to ascorbic acid. Carlic and carlosic acids are derivatives of *l*-γ-carboxymethyltetronic acid, also of *l*-γ-methyltetronic acid, and are structurally closely related to carolic and carolinic acids and also to ascorbic acid.[2] *l*-γ-Methyltetronic acid is also a product of *P. charlesii* G. Smith.[3]

Spiculisporic and minioluteic acids are complex fatty acids.

Table 90 shows the structural formulas of several acids formed by molds, while on page 349 some of the acids are enumerated.

PIGMENTS

Several different types of coloring matter have been isolated from molds. Hydroxyanthraquinones are produced by species of *Helminthosporium*. For example, a mixture of helminthosporin and catenarin constituted about 30 per cent of the dried weight of the mycelium of *H. gramineum* Rabenhorst, there being 2 to 3 parts of the former substance to 1 part of the latter. Cynodontin and tritisporin are other hydroxyanthraquinones produced by species of *Helminthosporium*. Certain dyestuffs may be manufactured from α-hydroxyanthraquinones.

[1] CLUTTERBUCK, P. W., H. RAISTRICK, and F. REUTER, *Biochem. Jour.*, **29**: 300–321 (1935).

[2] *Ibid.*, **29**: 871–883 (1935).

[3] *Ibid.*, **29**: 1300–1309 (1935).

TABLE 92.—SOME MOLD POLYSACCHARIDES PRODUCED FROM GLUCOSE[1]

Polysaccharide	Products of hydrolysis	Produced by
Capreolinose	Galactose, glucose, malonic acid, and mannose	*Penicillium capreolinum*
Galactocarolose	*d*-Galactose	*P. charlesii* G. Smith
Glycogen	Glucose	A white species of *Aspergillus*
Luteic acid	Glucose and malonic acid	*P. luteum* Zukal
Mannocarolose	*d*-Mannose	*P. charlesii* G. Smith
Mold starch	Glucose	*A. fumigatus, A. glaucus, A. niger, A. oryzae, P. glaucum, P. variabile*
Mycodextrin	Glucose	*A. niger, P. expansum*
Rugulose	Galactose	*P. rugulosum*
Varianose	*d*-Galactose, *d*-glucose, and either *d*-idose or *l*-altrose	*P. variens* G. Smith

[1] CLUTTERBUCK, P. W., *Jour. Soc. Chem. Ind.*, **55**: 55T (1936).

Thus it is within the realms of possibility that molds, utilizing glucose as the source of carbon, may sometime be used commercially in the production of dyestuffs.

Gould and Raistrick[1] isolated three crystalline pigments from species of the *Aspergillus glaucus* series. These included auroglaucin, an orange pigment; flavoglaucin, a yellow pigment; and rubroglaucin, a red pigment. Rubroglaucin has been shown to be a mixture of physcion, 4:5-dihydroxy-7-methoxy-2-methylanthraquinone, and erythroglaucin, a monomethyl ether of a tetrahydroxymethylanthraquinone.[2]

Ravenelin and rubrofusarin, two other pigments isolated from molds, are polyhydroxyxanthones.

Some data concerning mold pigments are given in Table 91.

SOME MISCELLANEOUS PRODUCTS

Acetaldehyde.—Using fixation methods, acetaldehyde has been recovered from media fermented by species of the genera *Aspergillus, Monilia, Mucor, Oïdium, Penicillium,* and other molds.

Ethyl Acetate.—This substance is produced by *P. digitatum* Sacc. from glucose media.

Ethyl Alcohol.—Although ethyl alcohol in small amounts is produced by species of several genera of molds, for example, *Aspergillus, Penicillium, Clasterosporium, Helminthosporium geniculatum,* and *Mucor race-*

[1] GOULD, B. S., and H. RAISTRICK, *Biochem. Jour.*, **28**: 1640–1656 (1934).
[2] ASHLEY, J. N., H. RAISTRICK, and T. RICHARDS, *Biochem. Jour.*, **33**: 1291 (1939).

mosus, it is produced commonly by species of the genus *Fusarium*. Yields of ethanol comparable to those obtained from yeasts have been produced by *F. lini* Bolley.[1]

The mechanism for the production of ethyl alcohol by fusaria has been studied by Anderson and his associates;[2] by White and Willaman;[3] by Nord;[4] by Gould, Tytell, and Hughes;[5] by Gould and Tytell;[6] and by others. The mechanism for the production of ethyl alcohol and carbon dioxide by fusaria is possibly similar to that of yeasts, according to Gould and Tytell, who have shown the presence of cozymase and a carboxylase and cocarboxylase system similar to that in yeasts.

Glycerol.—Glycerol has been produced by a white species of *Aspergillus*, by *A. wentii*, by a *Clasterosporium* species, by *H. geniculatum*, and by *M. racemosus*.

Methylglyoxal.—Sodium hexosediphosphate has been fermented by *A. niger* with the production of methylglyoxal (Suthers and Walker, 1932).

Chlorine-containing Compounds.—Erdin [$C_{15}H_7O_6Cl_2(OCH_3)$] and geodin [$C_{15}H_6O_5 \cdot Cl_2(OCH_3)_2$] are products of *A. terreus* Thom. Griseofulvin [$C_{17}H_{17}O_6Cl$] is a neutral, dextrorotatory crystalline substance produced by *P. griseo-fulvum* Dierckx.

Some Other Metabolic Products.—Table 93 supplies some information concerning fumigatin, mellein, spinulosin, and terrein.

MOLDS AND ARSENIC COMPOUNDS

Forty or more years ago the arsenic compounds present in the pigments of some old wallpapers and in some plasters were occasionally the cause of severe poisoning and even of fatalities. A fatal case of arsenic poisoning of this nature occurred in England as recently as 1931.[7]

A number of theories have been proposed to explain the cause of the poisoning. One of the earliest was that particles of the arsenic-containing pigment from the paper were inhaled. In 1839, Gmelin suggested that a volatile arsenic compound was responsible, since an odor of garlic was

[1] ANDERSON, A. K., and J. J. WILLAMAN, *Proc. Soc. Exptl. Biol. Med.*, **20**: 108 (1922).

[2] PRITHAM, G. H., and A. K. ANDERSON, *Jour. Agr. Research*, **55**: 937 (1937).

[3] WHITE, M. G. and J. J. WILLAMAN, *Biochem. Jour.*, **22**: 592 (1928).

[4] NORD, F. F., *Ergeb. Enzymforsch.*, **8**: 149–184 (1939). Abstracts of Communications, Third International Congress of Microbiology, New York, p. 337, Sept. 2–9, 1939.

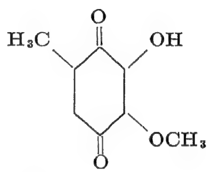
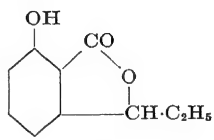
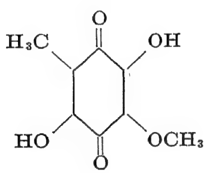
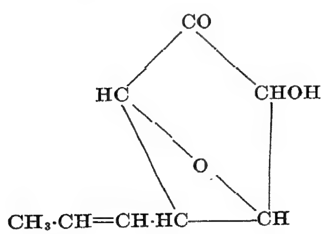
[5] GOULD, B. S., A. A. TYTELL, and W. L. HUGHES, JR., Abstracts of Communications, Third International Congress of Microbiology, p. 51, New York, Sept. 2–9, 1939.

[6] GOULD, B. S., and A. A. TYTELL. Unpublished data.

[7] CHALLENGER, F., *Jour. Soc. Chem. Ind.*, **54**: 657–662 (1935).

nearly always associated with the rooms where poisonings occurred. These rooms were commonly damp and moldy. Cacodyl oxide $[(CH_3)_2 \cdot As \cdot O \cdot As \cdot (CH_3)_2]$ was suggested by Basedow (1846) as being the substance concerned. One year later, Martin proposed that the gas might be arsine (AsH_3). The subject was carefully studied by Gosio (1891). Gosio exposed to the air potato mashes that contained arsenious oxide. Molds produced an odor of garlic from the potato mashes. One mold, which was named *Penicillium brevicaule* by Gosio, was particularly active in this respect. Other molds concerned were *Aspergillus glaucus*, *A. virens*, and *Mucor mucedo*. The gas produced by these molds from

TABLE 93.—SOME MISCELLANEOUS MOLD METABOLIC PRODUCTS

Product	Structural formula	Produced by
Fumigatin (3-Hydroxy-4-methoxy-2:5-toluquinone) ($C_8H_8O_4$)		*Aspergillus fumigatus* Fresenius[57] *
Mellein (A lactone of 6-hydroxy-2-α-hydroxypropylbenzoic acid) ($C_{10}H_{10}O_3$)		*A. melleus*[69] *A. ochraceus*
Spinulosin (3:6-Dihydroxy-4-methoxy-2:5-toluquinone) ($C_8H_8O_5$)		*Penicillium spinulosum* Thom[57,58,59]
Terrein (4-Propenyl-2-hydroxy-3:5-oxidocyclopentan-1-one) ($C_8H_{10}O_3$)		*A. terreus* Thom[42,54]

* The numbers in parentheses refer to the bibliography at the end of the chapter.

arsenious oxide, frequently referred to as "Gosio-gas," was shown by Challenger and his associates[1] to be trimethylarsine [$(CH_3)_3 \cdot As$].

Thom and Raper[2] have shown that *A. fischeri*, *A. sydowi*, and other organisms isolated from the soil may produce volatile substances from arsenic compounds.

Challenger and his associates have shown that several organic compounds of arsenic may be formed by certain molds. For example, methyldiethylarsine is produced from diethylarsonic acid [$(CH_3CH_2)_2$-$AsO \cdot OH$]; dimethyl-*n*-propylarsine from *n*-propylarsonic acid; and dimethylallylarsine [$(CH_3)_2As \cdot CH_2CH{:}CH_2$] from allylarsonic acid [$CH_2{:}CH \cdot CH_2AsO(OH)_2$] by the action of *P. brevicaule* Saccardo, grown on sterile bread crumbs.

Mixed alkylmethylarsines [$AsR(CH_3)_2$ and $AsR_2 \cdot CH_3$] are obtained with alkyl-arsonic and dialkyl-arsonic acids ($R \cdot AsO_3H_2$ and $R_2AsO \cdot OH$) from bread inoculated with *P. brevicaule*. Dimethyl selenide is produced from sodium selenate and selenite.

For other examples of methylating action, consult the papers by Challenger and his coworkers.

Qualitative Test for Arsenic.—Gosio[3] developed an extremely sensitive test for the detection of arsenic. The material suspected of containing arsenic was extracted with water or dilute acid. The extract was concentrated by evaporation of some of the water. Some of the concentrated extract was then added to a slice of potato that had been previously sterilized and inoculated with a culture of *P. brevicaule*, and the medium was incubated at 25 to 30°C. An odor of garlic would develop if a compound of arsenic was present. This test is said to be more sensitive then the Marsh test and to be able to detect as small a quantity as 0.000,001 g. of arsenious oxide in 1 g. of substance.

References on the Biochemistry of Molds

I. Studies by Raistrick and His Associates[4]

1. Raistrick, H., and W. Rintoul: I. Introductory, p. 2.
2. Birkinshaw, J. H., and H. Raistrick: II. Quantitative Methods and Technique of Investigation of the Products of Metabolism of Micro-organisms, p. 11.
3. ———, J. H. V. Charles, H. Raistrick, and J. A. R. Stoyle: III. Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products formed from Glucose by Species of *Aspergillus*, p. 27.
4. ———, A. C. Hetherington, and H. Raistrick: IV. Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products

[1] Challenger, F., C. Higginbottom, and L. Ellis, *Jour. Chem. Soc.*, p. 95 (1933).

[2] Thom, C., and K. Raper, *Science*, **76**: 548 (1932).

[3] Challenger, F., *Jour. Soc. Chem. Ind.*, **28**: 657–662 (1935).

[4] The first 18 of these studies were published in the *Philosophical Transactions of the Royal Society of London, Series B*, **220**: 1–367 (1931), under the title "Studies in the Biochemistry of Micro-organisms" by Prof. Harold Raistrick and his associates.

formed from Glucose by Species of *Penicillium* (including *Citromyces*), with an appendix by C. Thom, p. 55.

5. ———, ———, H. Raistrick, and J. A. R. Stoyle: V. Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products formed from Glucose by Species of *Fusarium*, p. 93.
6. ———, ———, A. C. Hetherington, and H. Raistrick: VI. Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products formed from Glucose by Miscellaneous Species of Fungi, p. 99.
7. , –, C. H. Lilly, and H. Raistrick: VII. On Kojic Acid (5-Hydroxy-2-hydroxy-methyl-γ-pyrone), p. 127.
8. ——— and H. Raistrick: VIII. The Estimation of Kojic Acid, p. 139.
9. ———, J. H. V. Charles, A. C. Hetherington, and H. Raistrick: IX. On the Production of Mannitol from Glucose by Species of *Aspergillus*, p. 153.
10. Raistrick, H., and W. Young: X. The Estimation of Mannitol in Fermentation Solutions, p. 173.
11. Hetherington, A. C., and H. Raistrick: XI. On Citromycetin, a New Yellow Colouring Matter Produced from Glucose by Species of *Citromyces*, p. 209.
12. Birkinshaw, J. H., and H. Raistrick: XII. On a New Methoxy-dihydroxy-toluquinone Produced from Glucose by Species of *Penicillium* of the *P. spinulosum* Series, p. 245.
13. Raistrick, H., and M. L. Rintoul: XIII. On a New Type of Mucilaginous Material, Luteic Acid, Produced from Glucose by *Penicillium luteum* Zukal, p. 255.
14. Hetherington, A. C., and H. Raistrick: XIV. On the Production and Chemical Constitution of a New Yellow Colouring Matter, Citrinin, Produced from Glucose by *Penicillium citrinum* Thom, p. 269.
15. Coyne, F. P., H. Raistrick, and R. Robinson: XV. The Molecular Structure of Citrinin, p. 297.
16. Clutterbuck, P. W., H. Raistrick, and M. L. Rintoul: XVI. On the Production from Glucose by *Penicillium spiculisporum* Lehman of a New Polybasic Fatty Acid, $C_{17}H_{28}O_6$ (the Lactone of γ-Hydroxy-$\beta\delta$-dicarboxy-pentadecoic acid), p. 301.
17. Birkinshaw, J. H., and H. Raistrick: XVII. The Products of Glucose Metabolism Formed by Various Species of Fungi (*Helminthosporium*, *Clasterosporium*, etc.), p. 331.
18. ———, J. H. V. Charles, and H. Raistrick: XVIII. Biochemical Characteristics of Species of *Penicillium* Responsible for the Rot of Citrus Fruits, p. 355.
19. Anslow, W. K., and H. Raistrick: XIX. 6-Hydroxy-2-methylbenzoic Acid a Product of the Metabolism of Glucose by *Penicillium griseo-fulvum* Dierckx *Biochem. Jour.*, **25**: 39 (1931).
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23. ——— and H. Raistrick: XXIII. Puberulic Acid $C_8H_6O_6$ and an Acid $C_8H_4O_6$, New Products of the Metabolism of Glucose by *Penicillium puberulum* Bainier and *Penicillium aurantio-virens* Biourge, with an Appendix on Certain Dihydroxybenzene-dicarboxylic Acids, *Biochem. Jour.*, **26**: 441 (1932).

24. Clutterbuck, P. W., A. E. Oxford, H. Raistrick, and G. Smith: XXIV. The Metabolic Products of the *Penicillium brevi-compactum* Series, *Biochem. Jour.*, **26**: 1441 (1932).

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CHAPTER XXXV

TEXTILE MICROBIOLOGY

Textile Fibers.—Textile fibers may be classified as natural and artificial fibers. Natural fibers include plant, animal, and mineral fibers. Among the plant fibers are coir (from the coconut shell); cotton (seed hair); flax, hemp, jute, ramie, and sisal (bast fibers); and other fibers of less commercial importance. Silk and wool are the two most important fibers of animal origin but very different in their mode of origin and in composition. Asbestos fiber (a silicate of magnesium and calcium) is of mineral nature. Rayons (artificial silk) are artificial fibers of vegetable origin. Lanital is an artificial wool made from casein. The production of other new synthetic fibers is very actively pursued at the present time, one being the Nylon which has been developed by the duPont Company.

There are several kinds of rayon: acetate rayon, cuprammonium rayon, and viscose rayon. The latter two rayons are regenerated cellulose.[1] Acetate rayons are less affected by mildew than other rayons.

The fibers of plant origin consist largely of carbohydrate substances, while those of animal origin are proteinous or nitrogenous in nature.

Types of Microorganisms Found on Textile Fibers.—As would be expected, most natural fibers show some microbial association. Bacteria, molds, actinomycetes, and yeasts are consistently found on textile fibers. In the case of plant fibers, these organisms have their origin in soil, water, etc., and as germs ecologically associated with green plants or introduced during processes of separating the fiber from other tissues. The presence of a particular organism may or may not be significant, for most of the normally occurring microorganisms produce no injury under ordinary conditions. Animal fibers also show characteristic types of microbes, some derived from air and soil, others from the bodies of the fiber-producing animals.

Conditions Favoring the Action of Microorganisms.—After the fibers have been separated and commercially baled or packed together changes in moisture content may take place. The availability of moisture and food material, a suitable temperature and pH, and the absence of chemical antiseptics favor the development of fungi and bacteria on commercial fibers and also to some degree on textiles. The fibers are in a sense potential foods, and as such vary according to wetness or dryness, exposure to air, and other factors.

[1] ALEXANDER, J., *Ind. Eng. Chem.*, **31**: 630 (1939).

Types of Destruction Caused by Microorganisms.—Under adverse conditions microorganisms may produce any of the following unfavorable actions: reduction or destruction of the tensile strength of the fibers; discoloration of the fabric owing to the production of pigments, perithecia, spores, or chemical by-products that react adversely with dyes already present; or alteration of the pH of fibers, with the result that the affinity for dyes is changed and the finished fabric may lack luster.

Mildew.—Mildew is a term used to denote the growth of fungi on various substrates.

Tendering.—Tendering, as applied to textiles, implies a weakening of the fibers.

Some Textiles Affected by Microorganisms.—Damage may occur in the raw fibers or in the finished products. Dyed and printed cotton goods, woolen goods, awnings, tarpaulins, tents, fishing nets, and many other articles are subject to the deleterious action of microorganisms unless they are kept dry or treated with chemical agents or otherwise protected.

Literature on the Microbiology of Textiles.—Thaysen and Bunker[1] have reviewed the literature concerning the microorganisms found on cotton and cotton textiles to the year 1927. Much of this literature and many other publications along these lines have appeared in the *Journal of the Textile Institute*. Prindle more recently has carried out extensive research on the microbiology of textile fibers, under the direction of Dr. S. C. Prescott. The results of this research, which is concerned principally with cotton and wool microbiology, have been published as a series of articles in *Textile Research*.[2] Prindle has reviewed briefly a large number of significant papers.

Excellent discussions of the microbiology of cellulose will be found Waksman's text on "Soil Microbiology" and in Thaysen and Bunker's text on "The Microbiology of Cellulose, Hemicelluloses, Pectin and Gum." It is obvious that any thorough study of textile microbiology must also involve a study of the microorganisms that attack cellulose.

COTTON

Structure.—The X-ray spectrometer, ultramicroscope, and chemical tests have established much new information in respect to the structure and composition of fibers.

The mature cotton fiber is hollow and twisted. It is coated with oil and wax, making it impervious to moisture. The individual cotton fiber is made up of fibrils, interlaced chains of elliptical cellulose micelles

[1] THAYSEN, A. C., and H. J. BUNKER, "The Microbiology of Cellulose, Hemicelluloses, Pectin and Gum," Oxford University Press, New York, 1927.

[2] PRINDLE, B., The Microbiology of Textile Fibres, *Textile Research*, 1933–1936.

(approx. 1.1 by 1.5 microns) embedded in a colloidal matrix of pectin nature.[1] The physical structure of the cotton fiber has been investigated by Farr,[2] by Farr and Eckerson,[3] and others.

Approximately 84 per cent of raw cotton is pure cellulose.

Numbers and Kinds of Molds and Bacteria.—Commercial raw cotton is usually highly infected with molds and bacteria.[4] Fresh samples of raw cotton fiber have yielded from 4 to 58 million bacteria and from 120,000 to 400,000 molds per gram.[4]

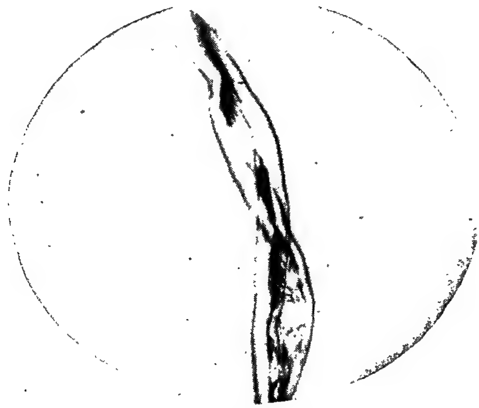

FIG. 49.—Cotton fiber. *(Courtesy The Textile Laboratory—Massachusetts Institute of Technology.)*

The molds and bacteria found on raw cotton were largely of the soil type or the type found on fresh plant tissues. Molds found on unstored samples[5] include species of *Hormodendrum, Fusarium, Alternarium, Sporotrichum* and *Monilia*-like organisms, with small numbers of the genera *Aspergillus* and *Penicillium;* bacteria included soil types in large numbers.

Stored samples of raw cotton contained aspergilli and penicillia, and soil types of sporeforming bacteria. Bacteria of the genera *Bacillus* and *Flavobacterium* predominated.[5]

Usually the aspergilli and penicillia isolated were able to utilize cellulose, starch, agar, and gelatin as the only sources of carbon, although rather slowly. The types of molds that predominated on fresh samples of

[1] ALEXANDER, *loc. cit.*

[2] FARR, W. K., *Jour. Applied Phys.*, **8**: 228 (1937).

[3] FARR, W. K., and S. H. ECKERSON, *Contrib. Boyce Thompson Inst.*, **6**: 189, 309 (1934).

[4] PRINDLE, B., *Textile Research*, **5**: 11 (1934).

[5] *Ibid.*

raw cotton grew more readily on cellulose, starch, gelatin, and agar (especially on the first three of these compounds), than the molds found in the stored samples.

According to Prindle, one would expect unstored samples to deteriorate much more rapidly than samples that had been stored.

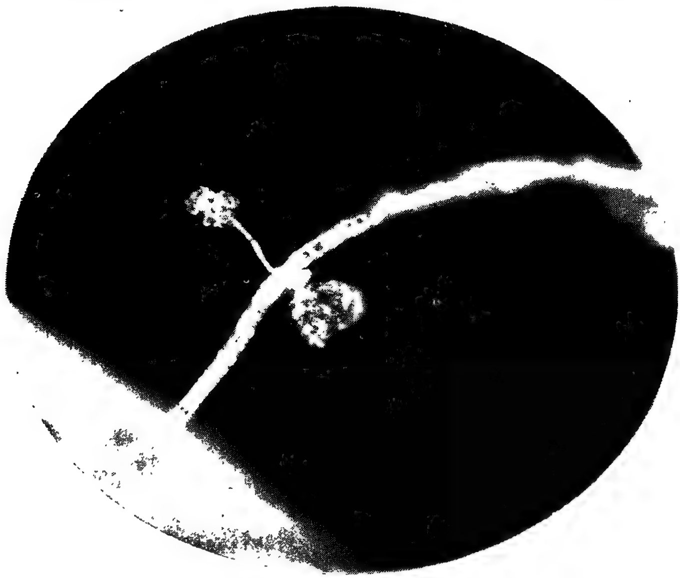

FIG. 50.—Fungus growth on cotton fiber. (*Courtesy The Textile Laboratory—Massachusetts Institute of Technology.*)

The bacteria found in raw cotton by Prindle were usually protein digesters, which did not demonstrate much action toward carbohydrate media, except for acid formation from glucose media. Hence it would not be expected that they would attack cellulose or starch appreciably.

Bacterial spores present in samples of raw cotton may survive the manufacturing processes. It is believed that many less resistant bacteria may also survive.

Fleming and Thaysen[1] found cotton deterioration in damp storage to be caused by a streptothrix and a cellulose-decomposing schizomycete.

Searle,[2] while investigating the rotting of textiles by microorganisms, found that *Stachybotrys* sp. was the most commonly occurring mold.

[1] FLEMING, N. and A. C. THAYSEN, *Biochem. Jour.*, **14**: 25 (1920).

[2] SEARLE, G. O., *Jour. Textile Inst.*, **20**: T162 (1929).

Galloway[1] has described 180 molds isolated from injured fabrics. He states that raw cotton is a source of much of the infection of cloth, and that some of the infection, for example, *Aspergillus niger*, may come from the boll.

TABLE 94.—BACTERIAL AND MOLD CONTENTS OF SEED COTTON, RAW COTTON, AND STORED COTTON[1]

	Seed cotton, 4 samples	Raw cotton, 12 samples	Stored cotton, 5 samples
Bacteria per gram:			
Highest count....	620,000,000	20,000,000	830,000
Lowest count....	27,000,000	95,000	38,000
Average.........	220,000,000	7,900,000	67,000
Molds per gram:			
Highest count....	1,150,000	480,000	2,250
Lowest count....	50,000	<3,000	65
Average.........	565,000	109,000	687

[1], B., *Textile Research*, **5**: 542 (1935).

According to Trotman and Sutton,[2] *Bacillus subtilis* and *B. mesentericus* may grow on cotton containing more than 9 per cent of moisture and cause deterioration of the fibers. (Neither of these organisms is able to decompose cellulose.)

Bacteria may cause the destruction of fibers and fabrics, even though fungi are not present. When damp cotton is baled, anaerobic cellulose-decomposing bacteria are likely to become active, especially in the center of the bale.

Microorganisms may be picked up during the growth, harvesting, transportation, and manufacture of cotton. Ginning and mechanical cleaning operations frequently do not materially reduce the numbers of microorganisms, and it is possible for mold spores to survive through the processes of spinning, sizing, and weaving.

Resistance of Different Cottons to Deterioration.[3]—Thaysen and Bunker have presented experimental data that indicate that cottons may vary considerably in resistance to microbiological deterioration. American cotton was most resistant, Egyptian cotton was less so, and India cotton decayed most rapidly. Thaysen and Bunker believe that the difference in resistance was due at least partially to differences in climate and soil conditions.

Fishing Nets.—Bacteria are responsible for a large percentage of the damage to fishing nets. As a result of this destructive action, consider-

[1] GALLOWAY, L. D., *Jour. Textile Inst.*, **21**: T277 (1930).
[2] TROTMAN, S. R., and R. W. SUTTON, *Jour Soc. Chem. Ind.*, **43**: T190 (1923).
[3] THAYSEN, A. C., and H. J. BUNKER, *Biochem. Jour.*, **18**: 140 (1924).

able research has been carried out by the U.S. Bureau of Fisheries and other agencies to develop antiseptics to protect the nets from rotting. Copper salts have been widely used as antiseptics for nets.

The Examination of Textile Fibers and Fabrics for Damage.—There are several methods for examining textiles or textile materials for evidences of damage. These include the use of the hand lens, the microscope, culture media, tensile-strength tests, viscosity tests, and other procedures. In conjunction with the use of the microscope, various staining agents have been used, alone and together with swelling reagents.

Color production, due to spores, perithecia, or pigments, or the musty odor frequently makes it possible to distinguish fungus growth on fabrics without more precise examination.

The microscope has given much valuable information[1] concerning the structure and condition of fibers. The use of stains, such as Victoria Blue B, in combination with swelling reagents has yielded particularly good results.

Victoria Blue B Stain.[2]—A 0.15 per cent aqueous solution of Victoria Blue B is added to the sample of cotton in the proportion of approximately 15 cc. of dye solution per 0.1 g. of sample, an excess of dye thus being assured. The sample is boiled for 1 min. in the dye, washed with cold water until no further color is removed, boiled with distilled water until bleeding of the color ceases, rinsed with cold water, permitted to drain, and then dried on a piece of filter or blotting paper.

Swelling Tests.—There are three swelling tests that have often been used to determine details of the structure of cotton as well as to obtain evidence of damage. These include the carbon bisulphide-sodium hydroxide test of Fleming and Thaysen,[3] the cuprammonium test, and the "critical" sulphuric acid test.

THE SWELLING TEST OF FLEMING AND THAYSEN.[4]—A uniform sample of the fibers is prepared by first carefully mixing 3 g. of the material. To approximately 0.2 g. of the sample are added 10 cc. each of carbon bisulphide and of 15 per cent sodium hydroxide solution. The sample and chemical reagents are shaken occasionally, while soaking of the fibers or hairs proceeds until the required swelling has been obtained (ascertained by examining small samples microscopically from time to time to determine when the swelling has reached an optimum point). Then three samples, each about the size of a large pea, are placed on three glass slides. The sample on each slide is mixed carefully, and approxi-

[1] BRIGHT, T. B., *Jour. Roy. Microscop. Soc.*, p. 141 (1925).

[2] PRINDLE, B., *Textile Research*, **6**: 481 (1936).

[3] FLEMING, N., and A. C. THAYSEN, *Biochem. Jour.*, **15**: 407 (1921).

[4] THAYSEN, A. C., and H. J. BUNKER, "The Microbiology of Cellulose, Hemicelluloses, Pectin and Gums," Oxford University Press, New York, 1927.

mately 20 fibers, selected at random, are "spread out horizontally" and covered with a cover glass, a drop of water being permitted to diffuse under the cover slip. The slide is examined microscopically. By scrutinizing three or more slides and making 10 counts on each slide for sound and damaged fibers, this test may be made quantitative in nature.

Normal cotton fibers present a beaded appearance when treated with swelling reagents. This result is due to the fact that the cuticles offer resistance to the expansion of the cellulose layers enclosed by them, while no resistance is offered to the layers between the cuticles. In damaged cotton hairs the cuticle has been injured or destroyed, while the cellulose has undergone some change. Consequently the beaded appearance found in normal cotton hairs is missing.

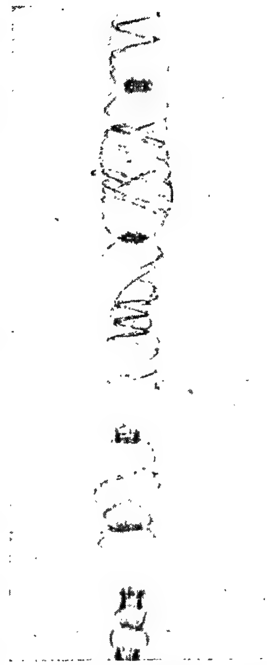

FIG. 51.—Cotton fiber swollen in sodium hydroxide and pressure. (*Courtesy The Textile Laboratory—Massachusetts Institute of Technology.*)

THE CUPRAMMONIUM SWELLING TEST.[1]—When this test is to be made, it is desirable to have all the necessary equipment close at hand and prepared. Forceps, needles, clean glass slides, cover slips, cuprammonium solution, and microscope should be available.[1]

The cuprammonium solution is prepared in the following manner: In 100 cc. of distilled water are dissolved 15.7 g. of copper sulphate ($CuSO_4 \cdot 5H_2O$). About 25 cc. of 6 N sodium hydroxide are added to this solution in order to precipitate the copper as copper hydroxide. The copper hydroxide mixture is filtered, and the hydroxide is freed of the sulphate ion by washing it with water. Finally the washed precipitate of copper hydroxide is dissolved in 500 cc. of 15.5 per cent ammonium hydroxide, and the resultant solution is stored in a brown glass bottle, which should be kept tightly stoppered.

The sample to be examined, which may be previously stained with Victoria Blue B and dried according to the procedure of Dr. Prindle, is slightly dampened by breathing onto it. It is placed on the top surface of a clean glass slide that has also been breathed upon (reducing the tendency of the fibers to fly about). A few of the fibers are pulled from the sample with the forceps and placed parallel to the long edge of the slide.

[1] PRINDLE, B., *Textile Research*, **6**: 481 (1936).

A few drops of cuprammonium solution from a partly filled bottle are added to the fibers, and a cover slip is placed over them. The mount is examined with a 100 × magnification. The cuprammonium solution will be too concentrated for most samples of cotton. Therefore small

FIG. 52.—Typical appearance of mildewed fiber under dissecting objective, showing general absence of strictures caused by resistant cuticle and the bunching of fibers which were parallel before swelling. [*Courtesy B. Prindle, Textile Res.*, **6**: 481 (1936).]

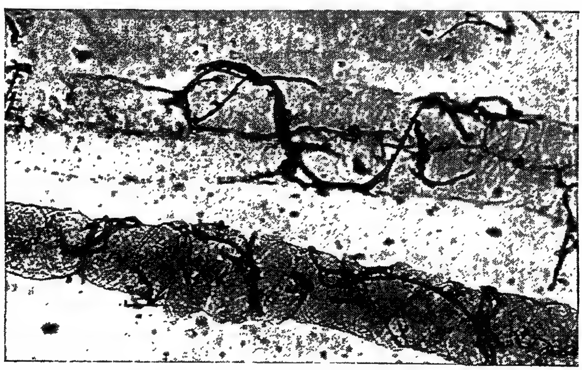

FIG. 53.—Appearance of mildewed fiber under 16-mm. objective showing absence of cuticle. Material in lumen is stained, and fibrillar structure is easily made out. The large black fibrous objects are possibly mold hyphae stained with Victoria Blue B but not swollen by the cuprammonium. [*Courtesy B. Prindle, Textile Res.*, **6**: 481 (1936).]

quantities of distilled water are added to the bottle containing the cuprammonium solution to reduce the concentration. The additions should be made gradually, the effect of a given concentration of the solution on the swelling rate being observed after each addition. In this manner, the proper dilution of cuprammonium solution for the sample being examined may be determined.

The correct concentration having been discovered, the fibers (placed in a parallel position on the slide) are covered with a few drops of the solution, and a cover slip is placed over the fibers plus the solution in such a manner that no air bubbles will be entrapped. The rate and nature of the swelling should be observed immediately with the microscope.

Normal samples of undamaged raw cotton are usually stained to an even dark purple-blue by the Victoria Blue B, according to Prindle, while mildewed samples exhibit a mottled appearance with "a lighter and clearer blue." It is believed that this color change is due to the fact that the pH of the dye has been altered by the acid nature of the mildewed area. This difference in the staining by Victoria Blue B is very helpful in distinguishing mildewed spots on fiber from normal fiber.

The swelling of the fibers is similar to that which takes place in the Fleming and Thaysen test, *i.e.*, normal fibers demonstrate a beaded appearance at a low magnification of the microscope.

Cuprammonium solution may be prepared readily and stored for long periods without loss of value. It is easy to control the concentration of the solution which is directly related to the rate of swelling of the sample.

The combination of staining with Victoria Blue B and the use of the cuprammonium reagent reveals much information concerning the condition of the fiber and details of the material contained in the lumen and the cuticle.

Research carried out by Küsebauch[1] using the Victoria Blue B stain followed by the use of the cuprammonium reagent confirms the observations made independently by Prindle.

Safranine-methylene Blue Stain for Mold Filaments in Cotton Fibers.—The sample of cotton is wetted with 95 per cent ethanol, stained for 1 min. with Loeffler's alkaline solution of methylene blue, washed with water, counterstained with safranine for 10 sec. or less, washed with water, dried, and examined with the low and high magnification of the microscope.

Pianese IIIb Stain.[2]—For the detection of mold mycelium in mildewed cotton fabrics, Jennison recommends the use of Pianese III*b* as a differential stain. The sample is washed in 95 per cent ethanol, stained for 15 to 45 min. with Pianese III*b*, washed with water, decolorized with acid alcohol, mounted on a glass slide, and examined microscopically.

The Congo Red Test.[3]—The Congo red test may be used to estimate the damage caused to fibers by microorganisms, heat, acid, and mechanical means.

[1] Küsebauch, K., *Melliand Textilber.*, **17**: 18 (1936).
[2] Jennison, M. W., *Science*, **72**: 346 (1930).
[3] Bright, T. B., *Jour. Textile Inst.*, **17**: T396 (1926).

The Congo red test is as follows: Approximately 0.1 g. of cotton is put into a suction flask with water. As much of the air as possible is evacuated. The cotton, after thus being wetted, is gently pressed to get rid of most of the water. It is placed in 25 cc. of an 11 per cent sodium hydroxide solution, shaken carefully, and permitted to stand for 5 min. The sample of cotton is quickly washed in water, placed in a saturated solution of Congo red (about 2 per cent), shaken intermittently for 6 min., removed, and washed with water until the water no longer shows any pink color. It is placed in an 18 per cent solution of sodium hydroxide. A few hairs are teased from the sample and mounted in the hydroxide solution on a glass slide. They are examined microscopically.

Undamaged cotton hairs are stained pink. Cotton hairs damaged by fungi are stained red and may be cracked. Such damage is nearly always associated with the presence of at least traces of the organism. Mechanically damaged hairs show bruises and cuts stained deeply. Hairs damaged by heat may show red spiral bands and singed cuticles. Injury caused by sulfuric acid results in irregular patches stained red.

Test for Mildew Resistance of Outdoor Cotton Fabrics.[1]—This test is designed to estimate the efficiency of the processes used to increase mildew resistance of cotton fabrics for outdoor use. *Chaetomium globosum* was selected as the test organism principally on account of its very destructive action on cotton fabric, its ease of cultivation, and its ability to grow under a variety of conditions. The test is as follows:

A sample of the fabric, sufficiently large to yield 12 strips, each 4 in. long in the direction of the warp and 1.25 in. wide, is soaked in running water, or in changes of water, for 2 days in order to extract water-soluble antiseptics that would be removed normally by rain or other weather conditions. The sample is air dried and cut into at least 12 strips of the dimensions stated. Five of these strips, which are to serve as controls, are put into one bottle, the remainder into a second bottle. The bottles with their contents are sterilized in an autoclave at a pressure of 15 lb. per sq. in. for 30 min. After cooling, the strips are ready for the test.

A special agar medium is prepared, which contains 3 g. of sodium nitrate; 1 g. of dipotassium hydrogen phosphate; 0.25 g. of potassium chloride; 0.25 g. of magnesium sulphate ($MgSO_4 \cdot 7H_2O$); 0.01 g. ferrous sulphate; 10 g. of agar, and 1,000 cc. of distilled water. The pH of the medium is adjusted to 6.8 and autoclaved for 20 min. at a pressure of 15 lb.

Agar is poured to a depth of 3 mm. in sterilized petri dishes that are 120 mm. in diameter and 15 mm. deep. At least seven plates are required for a test.

[1] THOM, C., H. HUMFELD and H. P. HOLMAN, *Am. Dyestuff Rpt.*, Oct. 22, 1934.

The sterile strips of cotton fabric may be infected with the spores of *Chaetomium* in one of two ways. They may be shaken in a sterile container with 10 cc. of a spore suspension, and then placed, one each, in separate petri dishes containing agar, using aseptic technique. In the alternate method, 1 cc. of the spore suspension is evenly distributed over the surface of the solidified agar in a petri dish, and then a strip is aseptically placed over the infected agar.

Since the agar medium contains no assimilable carbon, the cellulose of the fabric must serve as the source of energy.

The plates are incubated for 14 days at a temperature of 28 to 30°C. At the end of this period, the five strips that exhibit the most even covering with mold growth are washed to free them of agar and mold, and air dried.

The strips that have been subjected to mold action and the controls are raveled down in order that each strip may contain the same number of threads, corresponding to 1 in. of the original sample (a thread counter is advocated for this purpose). These strips are then placed in a chamber of constant temperature and humidity, for 2 days, in order to obtain results comparable with other tests carried out at various other times. The tensile strength of the fabric is then determined by a standard apparatus.

WOOL

Structure.—The wool fiber contains two principal layers of cells: the scales on the exterior and the cortex on the interior. Occasionally there is a trace of a medullary layer, which is always present in the hair fiber. The scales overlap one another, similar to those of a fish, and usually are highly serrated and loosely attached. Scales of hair are set more tightly to the cortex. Softness, ability to felt, and luster are related to the arrangement and kinds of scales. The cortical cells are fibrous and elongated. They impart strength and elasticity to the fiber, while the size and nature of these cells govern the waviness of the fiber.

Chemical Composition.—Chemically wool is made up largely of proteins. Keratin, which is found in hair, nails, hoofs, and horns, is the principal one. The average chemical composition varies with the type of animal, food, etc. On hydrolysis, keratin yields at least 16 different amino acids, of which glutamic, leucine, arginine, and aspartic acids are present in relatively large quantities. Sulphur is present to the extent of about 3.5 per cent in average wool, being a constituent mainly of cystine.

Speakman, Goddard and Michaelis, Ashbury, and others have studied the structure and composition of the wool fiber.[1]

[1] SPEAKMAN, J. B., *Jour. Soc. Dyers Colourists*, Jubilee Issue, 1934; GODDARD,

Several substances are usually found deposited on the surface of the wool fiber. These include wool greases (compare with lanolin); water-soluble compounds, such as potassium soaps and potassium salts, urea, etc.; soil; and other matter. The matter thus found on wool is known as "yolk."

FIG. 54.—Wool fiber. [*Courtesy The Textile Laboratory—Massachusetts Institute of Technology.*)

Microbiology.—The microbiology of wool has been investigated by various workers. Burgess, Trotman and Sutton, Bright, Galloway, Prindle, and others are outstanding in their contributions to this phase of textile science.

Molds, actinomycetes, bacteria, and yeasts have been found on wool and may bring about undesirable changes, which include discolorations and deterioration of the fibers.

Prindle[1] has isolated, among the molds, species of *Alternaria*, *Stemphylium*, *Oöspora*, and *Penicillium* that completely or partially destroy the structure of wool. Species of *Alternaria*, *Stemphylium*, and *Oöspora* were particularly destructive and productive of discolorations. Other molds capable of altering the structure of wool include species of *Aspergillus*, *Dematium*, *Fusarium*, *Trichoderma*, and *Cephalothecium*.

Of the bacteria, several aerobic bacilli have been found to have the ability to deteriorate wool, while a few cocci caused discolorations (pink to red), without injuring the strength of the fiber. Nonsporeforming rods also have produced discolorations, which may be red, yellow, orange, or of other color. The alkaline nature of fleece favors the growth of some types of bacteria rather than that of molds.

The scouring and drying of wool during processing removes or inactivates a large part of the molds and nonsporeforming bacteria, but aerobic bacilli survive.

Sterilization of Wool.—Humfeld, Elmquist, and Kettering[2] have studied several methods for sterilizing wool fabrics in an effort to deter-

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D. R., and L. MICHAELIS, *Jour. Biol. Chem.*, **106**: 605 (1934); ASHBURY, W. T., *Nature*, **140**: 968 (1937).

[1] PRINDLE, B., *Textile Research*, **5**: 542; **6**: 23 (1935).

[2] HUMFELD, H., R. E. ELMQUIST, and I. H. KETTERING, *U.S. Dept. Agr.*, *Tech. Bull.* 588, September, 1937.

mine the most satisfactory one for use in making subsequent bacteriological studies, using *Bacillus mesentericus* as the test organism. They found that wool fabrics could be heated in xylene, Stoddard solvent, or tetrachlorethylene satisfactorily, spores being destroyed and the fabrics being undamaged. The method consisted of heating the fabrics with xylene for 12 hr. at 100°C., or for 2 hr. at 121°C., or for 1 hr. at 134.5°C. No bacteriostatic effects were noticed in fabrics thus treated.

Physical and chemical properties of wool were altered by the use of intermittent steaming and wet or dry autoclaving.[1]

Ultraviolet light, iodine, potassium permanganate, alcohols, and certain other agents failed to produce sterility of the wool under the conditions of the experiment.

Formaldehyde and mercury salts produced sterility of the fabrics but were retained to some extent.

For further details consult the bulletin cited.

Sieber's Test for Damaged Wool.—A sample of wool is degreased by washing it first in ether, then in water. The washed sample is boiled for a few minutes in a 1 per cent solution of Benzopurpurine 10 B. The dyed sample is boiled with water until no more color is extracted. It is then examined with a microscope. Those parts of the wool which have been damaged mechanically by acid or alkali will be stained red.

Fiber vs. Fabric.—The foregoing pages have dealt with fibers most commonly used in textile manufacturing. In making and finishing many types of the fabrics now on the market two or more classes of fibers may be employed, and, furthermore, the woven goods may be subjected to a variety of processes such as sizing (with starchy materials), bleaching, dyeing, and use of finishers. These processes may exert an effect on the microbial populations attached to the fibers or may change the life-supporting character of the finished goods. From this standpoint, probably the most important of the processes named is the use of sizing materials, which in themselves may introduce new infection with microbes, and which are not always completely removed by the diastatic preparations employed as desizing agents.

The Prevention of Growth of Microorganisms on Textiles.—There are two principal methods for preventing the growth of microorganisms on textile fibers and fabrics. The first and the only sure method (thus far known) consists of reducing the moisture content of the goods to less than 8 per cent and maintaining it below this maximum. Galloway[2] has shown the importance of relative humidity in relation to the storage of fabrics. He stated that the "safe" figure for storage depended on the microorganism instead of the nature of the material. Thus the minimum relative humidity that permitted the growth of several types

[1] *Ibid.*

[2] GALLOWAY, L. D., *Jour. Textile Inst.*, **26**: T123 (1935).

of molds varied between 75 and 95 per cent. Certain species of *Aspergillus*—for example, *A. glaucus*, *A. candidus*, and *A. versicolor*—were able to grow at relative humidities of 75 to 80 per cent. Thus, in order to prevent mildew it is necessary to maintain a relative humidity that is too low to permit growth.

Obviously it is impossible to keep dry such articles as tarpaulins, tents, and fish nets. The second method indicates the use of a suitable antiseptic. Although fairly satisfactory chemical agents have been tried as textile antiseptics, the search for the ideal antiseptic still continues. Many substances have been proposed, as is evidenced by the large number of publications dealing with this subject.

Morris and others[1] have outlined the properties of the good antiseptic. The good antiseptic (1) must be sufficiently soluble in water to mix evenly with the size; (2) must be stable to heat (for example, during the boiling of the size), drying, and oxidation; (3) should be odorless at various pH values; (4) should be free of color and should not alter that of the fabric; (5) must not injure the fabric; (6) must not injure metal attachments or machinery; (7) must not affect dyeing and finishing operations; (8) must not alter the size and thus affect the "feel" of the fabric; (9) must be safe to handle, and (10) must be readily available in large quantities and at a low cost.

Tarpaulins, tent canvas, awnings, roofing paper, and fishing nets may be preserved from the action of microorganisms by the application of antiseptics. Copper compounds have been widely used, especially to treat fish nets. Copper oleate, as well as copper oxide and mercuric oxide, or mixtures of these, have been recommended by Conn.[2]

According to Taylor and Wells,[3] copper oleate is particularly effective when applied a second time to a fishing line that has been immersed for some time after the first treatment. Copper compounds add color to the fabrics being treated, and for this reason their field of application is limited.

Zinc chloride has been used extensively in sizes, but it is a heavy compound and not desirable for certain fabrics. Benzoates, borates, complex fluoride salts, salicylates, and many other chemical compounds have been used with variable results.

Oil of thyme, particularly when mixed with turpentine and rosin oil, has been used as a disinfectant for carpets (Funch-Hellet), and for other purposes.

[1] Morris, L. E., *Jour. Textile Inst.*, **18**: T99 (1927); Fargher, R. G., L. D. Galloway, and M. E. Robart, *Jour. Textile Inst.*, **21**: T245 (1930); Roberts, H. C., *Am. Dyestuff Rpt.*, **19**: 431 (1930).

[2] Conn, W. T., *U.S. Bur. Fisheries Doc.* 1075, 1930.

[3] Taylor, H. F. and A. F. Wells: *U.S. Bur. Fisheries Docs.* 947 (1923) and 998 (1925).

Galloway has recommended the use of *p*-chloro-*m*-cresol for finishing baths. He has suggested also (1930) the use of carbon dioxide as a storage gas.

Proctor has shown that fibers inoculated with *Aspergillus niger* and then treated with a mixture of ethylene oxide gas and carbon dioxide did not evidence mold development when subsequently exposed to the air. This suggests that storage of finished fabrics in chambers provided with these inhibitory gases may be commercially applied.

For an extensive review of this subject, the student is referred to the textile journals, patent literature, and other publications.

RETTING

Purpose.—The fibers of flax and hemp (bast fibers) are commonly loosened from the stems that contain them by a process known as "retting." This is an ancient term meaning "soaking in water." The fiber bundles of flax lie between the soft-walled cells[1] of the cortex and of the

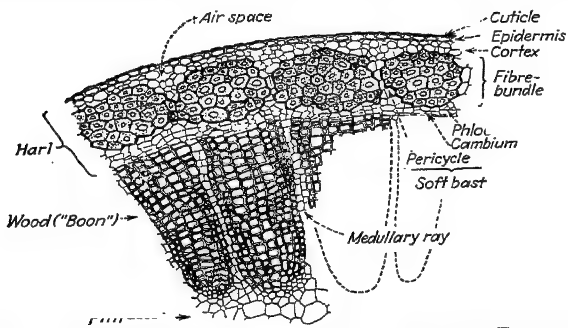

Fig. 55.—Diagrammatic cross section of a flax stem. [*Courtesy Eyre and Nodder, Jour. Textile Inst.*, **15**: T239 (1924).]

central wood. Retting, if efficient, should accomplish a satisfactory loosening of the fiber bundles from the cortex and wood and effect a partial digestion or loosening, at least, of the cementing material between the various fiber bundles.

Methods.—Retting is accomplished by both anaerobic and aerobic methods. Anaerobic methods include those in which retting is carried out in flowing or in stagnant waters. For example, the Egyptians have for hundreds of years retted flax in the soft, warm, slow-flowing waters of the River Nile under almost ideal conditions. Retting in slow-flowing waters has also been practiced in Belgium, Germany, and Holland; while Italy and Ireland have made use of stagnant waters, such as ponds and dams. Aerobic methods may include those in which the material

[1] Eyre, J. V., and C. R. Nodder, *Jour. Textile Inst.*, **15**: T237 (1924).

is retted in vats supplied with aeration or the method known as the "dew" or "land-retting" method.

Retting is accomplished in the anaerobic methods principally as the result of bacterial action; in the aerobic dew method, largely by the action of fungi.

Selection of Method.—The choice of method will depend on several factors: the agricultural situation, the nature and quantity of the available straw, the water supply, the climate, the costs involved, and other factors.

Anaerobic Retting.—The following comments will be confined to a general discussion of retting as carried out by anaerobic processes, whether in a vat, a river, or other place.

Retting may be considered to take place in three stages: a physical stage; a biological stage, which may be subdivided into preliminary and principal phases; and a mechanical stage.[1]

1. *Preliminary.*—It is important to prepare the material carefully for retting. For example, in the deseeding of the flax plant, damaging of the fibers leads to the evils accompanying overretting, *i.e.*, weakening, discoloration, etc. The raw material should be carefully sorted and treated according to its nature and condition.

The chemical changes that take place will vary according to the types of microorganisms that predominate, the nature of the water, the material being retted, and the method used.

2. *Physical Stage.*—During the physical stage, water is absorbed by the tissues of the stem, swelling occurs, water-soluble substances are extracted, and bacteria develop. Fissures and breaks frequently appear in the woody portion, while bubbles of air are given forth from the tissues. The substances extracted, which amount roughly to 12 per cent, include sugars, glucosides, tannins, soluble nitrogenous constituents, and coloring matter. The surrounding liquid thus becomes a highly colored medium for the development of bacteria and other microorganisms.

3. *Biological Stage.*—During the preliminary biological stage, many of the bacteria and other microorganisms, which are resident on the materials being retted, grow and multiply. Aerobic forms predominate at first, since the water contains dissolved oxygen and nutrients favoring their development. Yeasts and molds may develop on the surface of the water. In using up the dissolved oxygen, the aerobic organisms tend to establish anaerobic conditions. Organic acids and gases, especially carbon dioxide, are produced.

Actually retting occurs mainly during the principal biological stage. The middle lamella is softened, cells of the plant tissues are separated, and the connections between the bundles become weakened.

[1] THAYSEN and BUNKER, *loc. cit.*

The anaerobic organisms responsible for retting multiply rapidly during this period. These include the bacteria, which elaborate the enzymes capable of hydrolyzing the pectin of the middle lamella of the parenchymatous bark tissue, thus causing a separation of the fiber bundles from the cortex and wood. *Plectridium pectinovorum* (related to *Clostridium butyricum* Prazmowski[1]) and *Cl. felsineum* (*Bacillus felsineus*) are two anaerobic pectin-dissolving bacteria of considerable value in retting processes. Ruschmann[2] considers *B. amylobacter* (also related to *Cl. butyricum* Prazmowski) to be the most important anaerobic organism in the warm-water vat process. Makrinov[3] used pure cultures of *Pectinobacter amylophylum* with "superior retting results."

Various substances are produced during the fermentation, depending on the organisms and the conditions of the retting operation. These may include organic acids, such as acetic and butyric acids; gases—carbon dioxide, hydrogen, and sometimes methane and hydrogen sulphide; solvents, such as ethanol, butanol, and acetone; and other substances. Lactic acid production interferes with the action of butyric acid bacteria, such as *B. amylobacter*. Organic acids may be oxidized at the surface of the vat to simpler compounds.

It is important not to overret flax, since the lignified pectin of the fiber bundles may be hydrolyzed with the result that individual fibers become separated.

Cellulose is not fermented by desirable retting organisms.

4. *Mechanical Stage.*—The retted material is washed (if in a vat, by an upward flow of water) to carry away portions of mold films, organic acids, odors, and other undesirable substances that may be associated with the plant material. Sometimes just sufficient alkali, in the form of soda, is added to neutralize the acids present in the vat (if one is used), resulting in an increase in the luster and suppleness of the fiber. Another method for freeing the material from organic acids after washing is to expose it to the action of aerobic bacteria.

The washed material is carefully dried by natural or artificial means.

Finally, the dried retted fiber tissue is separated from the cortical and wood residues by the use of machines.[4]

Temperature of Retting.—A fairly wide range of temperatures has been used in various retting processes. In general, it may be stated that

[1] "Bergey's Manual of Determinative Bacteriology," p. 749, 5th ed., The Williams & Wilkins Company, Baltimore, 1939.

[2] Ruschmann, G., *Jour. Textile Inst.*, **15**: T61, T104 (1924).

[3] Makrinov, I. A., Dnevnik Vsespziunogo, *Sez Botan. Leningrad*, **206**: 1928 (1928).

[4] Thaysen and Bunker, *loc. cit.*

higher temperatures, 37 to 38°C., for example, favor rapid retting but are sometimes less desirable from the viewpoint of the quality of the final product, for overretting is more likely to occur at higher temperatures and leads to the damaging of the fibers. Good results may be obtained, however, by carefully controlling the processes that use higher temperatures. The Carbone retting process employs a relatively high temperature.

Temperatures of 26 to 28°C. or 30 to 32°C. are considered to be most favorable for retting.[1]

Dew retting is subject to wide variations of the temperature.

Carbone Retting Process.—In this process, a mass culture of *Cl. felsineum* (*B. felsineus*) is prepared in a potato medium and added at the rate of 1 liter to 10 kg. of dry tissue to the water of the retting vat. The retting temperature is 37 to 38°C., the optimum for the growth of this organism. A period of 50 hr. or less is usually required for retting, but a longer time may sometimes be necessary. This process naturally requires closer supervision than certain other retting processes. The fibers produced are bright colored, while the yield is said to be good.

Aerobic Retting Processes.—An aerobic method of retting was developed by G. Rossi. A mass culture of *B. comesii* is added to the plant material in a vat. The water, maintained at 28 to 30°C., is aerated to favor the development of aerobic bacteria, *B. comesii* in particular. It has been stated that a smaller quantity of organic acid is produced by this process, while the danger of overretting is greatly reduced and fibers may be dried artificially without danger. According to Ruschmann, such fibers are fuller in appearance, darker, and harder.

Dew Retting.—The retting action of this process is due principally to molds, but bacteria are present in large numbers.

In carrying out the process, the material to be retted is spread out in thin layers on suitable vegetation. It is thus exposed to the action of the sun, dew, and rain. Atmospheric conditions, the retting bed, and the soil are mainly responsible for the quality of retting. As would be expected, there is a minimum of control used in this process, which is simple and inexpensive. Although good fiber may be obtained by this method, the fibers are frequently of a poor quality and the yields small.

Improvements in Methods.—In this very brief description of a limited number of retting methods, it has been impossible to discuss adequately the problems of the processes, which are concerned with the yield of the fiber and its quality—softness, strength, color, etc. It will suffice to state that improvements in procedures are constantly being made. A review of the literature of textile journals will yield information along these lines to the zealous student.

[1] Rüschmann, G., *loc. cit.*

Periodicals on Textile Microbiology and the Textile Industry

American Dyestuff Reporter (fortnightly), Howes Publishing Co., New York.
Bulletin of the U.S. Institute for Textile Research, Inc. (monthly), Boston.
Canadian Textile Journal (weekly), Canadian Textile Journal Publishing Co., Ltd., Montreal.
Cotton (weekly), Manchester Cotton Association, Ltd., Royal Exchange, Manchester, England.
Cotton (monthly), W. R. C. Smith Publishing Co., Atlanta, Ga.
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CHAPTER XXXVI

THE MICROBIOLOGY OF WOOD

Wood may be injured or destroyed by several agencies, causing great losses annually. Fire is obviously the most serious enemy. Violent windstorms may be the direct cause of extensive destruction. Wear and weathering are inevitable. Animals, such as mice and rabbits, may do great damage by girdling fruit or other trees and causing their death. Biological agents alone cause the destruction of many millions of dollars worth of valuable wood each year. Included among the biological agents are the fungi—the principal biological causes of wood breakdown and chemical destruction; the insects—among which the termites are outstanding in importance; and molluscan and crustacean borers. Certain insects and borers are especially significant in the impairment or ruination of structures built of timber such as warehouses, wharves, trestles, and domestic buildings. From the standpoint of industrial microbiology, the changes produced in wood by the attacks of fungi are especially significant.

Structure and Composition of Wood.—It is not within the scope of this text to discuss in detail the structure of wood. In general, wood may be regarded as the mass of cells fully developed and modified during tree growth. It consists principally of lignin,[1] cellulose, and hemicelluloses with small quantities of starch, protein, and mineral salts.

Lignin is a constituent of the cell wall, to which it imparts strength and rigidity. Its exact chemical structure is unknown, but it is believed that the essential grouping of the molecule is coniferyl alcohol, coniferyl aldehyde, or a compound of closely related structure.[2]

Cellulose, $(C_6H_{10}O_5)_n$, a polysaccharide made up of a chain of beta-glucose residues joined at carbon atom 4 by glucosidal linkage,[2] when hydrolyzed, gives rise first to cellobiose and then to glucose (see Chap. III).

[1] SCHORGER, A. W., "The Chemistry of Cellulose and Wood," McGraw-Hill Book Company, Inc., New York, 1926; HAWLEY, L. F., and L. E. WISE, "The Chemistry of Wood," Reinhold Publishing Corporation, New York, 1926; NORMAN, A. C., "The Biochemistry of Cellulose, Polyuronides, Lignin, Etc.," Oxford University P New York, 1937; PHILLIPS, M., *Chem. Rev.*, **14**: 103 (1934); HÄGGLUND, E., "Holzchemie," 2d ed., Akademische Verlagsgesellshaft m.b.H., Leipzig, 1939; FREUDENBERG, K., *Ann. Rev. Biochem.*, **8**: 81 (1939).

[2] GORTNER, R. A., "Outlines of Biochemistry," 2d ed., John Wiley & Sons, Inc., New York, 1938.

Hemicelluloses include hexosans and pentosans, such as mannan, galactan, xylan, and araban, which yield mannose, galactose, xylose, and arabinose, respectively, when hydrolyzed by dilute acids. Levulose is also produced from certain hemicelluloses by hydrolytic processes. The hemicellulose content of hardwoods is greater than that of softwoods.

One analysis of spruce (*Picea excelsa*)[1] showed 30 per cent of lignin, 53 per cent of cellulose (free from pentosans), 15 per cent of hemicelluloses, and 2 per cent of fat, protein, resin, etc.

Ritter[2] of the Forest Products Laboratory of the U.S. Department of Agriculture has carried out research concerned with the structure of the cell wall, which has been dissected into layers, fibrils, fusiform bodies, and spherical units. Most of the lignin is present in the middle lamella, the rest occurring in other parts of the cell wall which is largely made up of cellulose and hemicelluloses.

For a detailed discussion of the structure and chemistry of wood, the reader is referred to some of the publications listed at the end of the chapter.

THE FUNGI

The fungi here considered include certain forms of *Basidiomycetes* and *Ascomycetes* ("higher fungi") which actually bring about a disintegration of woody substances (decay, rot, and other types of breakdown); those which by pigment formation or by other means produce stains on timber, thus rendering the wood less valuable for some purposes; and "molds," including numerous forms of fungi imperfecti. Wood-destroying fungi are, of course, both helpful and harmful in the economy of nature. The breakdown of plant tissues, leaves, branches, and much fallen timber, either from natural or artificial causes, is advantageous, since the organic matter composing them is gradually decomposed and in large part returned to the soil as a result of the action of the enzymes manufactured by the fungi and the oxidations that follow. If timber were not so broken down, it would accumulate in useless masses and interfere with forest growth. On the other hand, wood-destroying fungi attack and seriously injure much valuable timber. Logs may be destroyed before there is an opportunity for yarding them. Sawed lumber, wooden ties, piles, sills, buildings, the supporting material of mine shafts, telephone and telegraph poles, fence posts, etc., are subject to the action of wood-destroying fungi, unless such wood is treated by chemical preservatives or otherwise to render it unsusceptible to attack.

[1] SCHORGER, *op. cit.*

[2] RITTER, G. J., *Paper Ind.*, June, 1934.

The action of wood-destroying fungi is favored by the presence of moisture, warmth, and the absence of direct sunlight, as well as by the presence of oxygen and nitrogenous food materials that may be utilized by the fungi. On the other hand, there are certain volatile oils and water extractives contained in some heartwoods, as, for example, in cedar, that are toxic to fungi and therefore prevent or restrain decay for long periods.

Knowledge of the growth conditions of these organisms is of prime importance in procedures designed to prevent the action of wood-destroying fungi. For example, most fungi will not grow in wood containing less than 20 per cent moisture (on the basis of the oven-dried weight). On the other hand, the fungi will not grow when timber is submerged in water, or deep in the soil, because there is a deficiency of oxygen. Destruction of wood proceeds slowly or not at all in the cold weather of our Northern states, or in buildings maintained at low temperatures, but proceeds very rapidly in hot moist climates. Several types of chemical agents are very useful in preventing the action of fungi and in destroying them and are quite widely employed.

Some Important Wood-destroying Fungi.—Almost all the fungi that destroy wood are members of a few families of the Basidiomycetes.[1] Fungi belonging to the following genera are possessed of the ability to destroy wood: *Collybia*, *Lentinus*, *Pholiota*, *Pleurotus*, and *Schizophyllum* of the family *Agaricaceae; Echinodontium* and *Hydnum* of the family *Hydnaceae; Daedalea*, *Fistulina*, *Fomes*, *Ganoderma*, *Lenzites*, *Merulius*, *Polyporus*, *Poria*, and *Trametes* of the family *Polyporaceae;* and *Coniophora*, *Hymenochaete*, *Peniophora*, and *Stereum* of the family *Thelephoraceae*.

Enzymes of Wood-destroying Fungi.—The enzymes secreted by the wood-destroying fungi are responsible largely for the complex chemical changes that take place when the wood is attacked. Those fungi which completely disintegrate wood must elaborate enzymes that will attack the cell walls (cytase), the lignin (ligninase), the cellulose (cellulase), the hemicellulose (hemicellulase), and the various other substances present or formed as intermediates in the breakdown process.

The kinds and quantities of enzymes elaborated will depend on the species of fungus, the nature of the substrate, the pH, the temperature, and other factors. The following are some of the enzymes that have been reported as secreted by various wood-destroying fungi:[2] amidase, amylases, asparaginase, catalase, cellulase, cytase, emulsin, erepsin, esterase, glucosidase, hemicellulase, hippuricase, inulase, invertase, laccase, lactase, ligninase, lipase, maltase, oxidase, pectinase, protease, raffinase, rennet, tannase, trypsin, tyrosinase, urease, and others.

[1] Boyce, J. S., "Forest Pathology," McGraw-Hill Book Company, Inc., New York, 1938.

[2] Bose, S. R., *Ergeb. Enzymforsch.*, **8**: 267 (1939).

Rots.—Two classes of rots are common in wood: the white rots, which may include pocket, stringy, flaky, or mottled rots; and brown rots, which include pocket, stringy, mottled, ring, or cubical rots.

White rots are produced chiefly by fungi that attack the lignin principally, leaving white areas made up of cellulose compounds, and to a lesser degree by fungi that attack the cellulose and cause bleaching of the lignin. White rots are especially addicted to Douglas firs but are found also in white pine, other conifers, hardwoods, mine timbers, etc. The red ring rot, whose causative agent is *Fomes pini* (*Trametes pini*), is a white pocket rot that attacks Douglas fir, ponderosa pine, and other woods. It does not attack wood that is in use. Red ring rot is the most serious cause of loss due to decay in this country.[1] It attacks the heartwood particularly. Hardwoods may be attacked by *Fomes applanatus* (the shelf fungus) when stored, or by *Fomes igniarius*.

Brown rots are commonly caused by cellulose-attacking fungi, although rots are not confined to these. The wood attacked by such fungi often may become so friable as to be pulverized by the fingers. Brown rot fungi may attack the sound wood which is found between areas attacked by white rot fungi. Brown rots are frequently found in building timbers.

Poria incrassata is the cause of brown cubical rot, a dry rot that destroys millions of dollars worth of coniferous timber in buildings in this country each year, especially in the states lying near the coasts of the Gulf and the Northwest. The fungus may transport moisture for several feet through its rhizomorphs. Thus it may attack and destroy wood which would otherwise remain dry. *Merulius lacrymans* produces a similar type of destruction of coniferous wood. This fungus, though common in Europe, is found infrequently in the United States. It also possesses rhizomorphs that may transport water for some distance. Its growth is not favored by high temperatures.

Brown rots of softwood are also caused by *Polyporus schweinitzii*, *Fomes pinicola*, *F. laricis*, *Trametes seriales*, and other fungi.

Wood-staining Fungi.—The staining of wood may be caused in general by two different agencies: chemical action, in which oxidizing enzymes are active in bringing about color changes in the sapwood; and fungi. The stains or discolorations produced by the fungi may be confined mainly to the surface of the woody materials, in which case they may be readily removed by planing or other treatment, or they may penetrate deeply into the wood rendering such removal out of the question.

Stains may appear on wood products, on logs, or on dead or dying trees. The stains are usually confined to the sapwood, although occasionally the heartwood is affected. The presence of suitable food, moisture, and an optimum temperature favors the production by fungi

[1] Boyce, *op. cit.*

of stains of various shades. For example, blue stains, which are very common, are produced by *Cerastostomella*, *Alternaria*, and other molds; a grayish-black stain is caused by *Torula ligniperda;* a green stain by *Chlorosphenum aeruginosum;* a red stain by *Fusarium negundi;* and a yellow stain by *Penicillium divaricatum.*

In general, species of the following genera may produce discolorations that may be removed by planing, the use of steel brushes, or some other method: *Alternarium, Aspergillus, Cladosporium, Fusarium, Graphium, Mucor, Penicillium, Rhizopus, Torula* and others. The stain or discoloration is usually produced as a result of the color formed in the mycelium of the mold, as a result of a soluble pigment, or as a result of a chemical reaction between some compound produced by the mold and the wood.

Staining may be prevented or controlled by drying the wood, by submerging it in water, by treating it chemically, or by other means. Drying of the wood is considered most effective; submergence is but a temporary measure. Occasionally wood may become stained before it can be dried. Resort may then be had to stain-preventing chemical dips. Boyce[1] states that a dip composed of a 0.24 to 0.36 per cent solution of 6.3 per cent ethyl mercuric chloride, or a 0.5 to 1 per cent solution of equal parts of sodium tetrachlorophenoxide and sodium 2-chloro-orthophenylphenoxide, is effective in treating southern pines or hardwoods. Hardwood and red gum may be treated with a 5 per cent solution of commercial borax.

Hubert,[2] as the result of an extensive investigation involving the control of stain and decay in finished wood products (such as doors, window frames, and millwork in general) which are exposed to considerable moisture in service, has selected and recommended as being promising four chemical compounds out of the 25 chemicals and 18 proprietary wood preservatives he has studied. These chemical compounds included pentachlorophenol, *o*-phenylphenol, 2-chloro-o-phenylphenol (Permatol *D*), and tetrachlorophenol. Hubert[3] has outlined the requirements for an effective preservative for exterior millwork, such as sash, door, and frame stock.

For further details connected with the destruction and staining of wood by fungi, the reader is referred to the works of the Forest Products Laboratory of the U.S. Department of Agriculture at Madison, Wis.; to the reports of the Bell Telephone System; to the texts listed at the end of this chapter—of which those by Boyce[1] and Hunt and Garratt[4] are

[1] Boyce, *op. cit.*

[2] Hubert, E. E., *Ind. Eng. Chem.*, **30**: 1241 (1938).

[3] Hubert, E. E., *Western Pine Assoc. Rev., Tech. Bull.* 6, Apr. 15, 1938.

[4] Hunt, G. M., and G. A. Garratt, "Wood Preservation," McGraw-Hill Book Company, Inc., New York, 1938.

particular interest; and to the publications of the American Wood-preservers Association and the various schools of forestry.

Examination of Wood for the Presence of Fungi.—Wood may be examined for the presence of fungi by macroscopic, microscopic, cultural, and other methods. Discolorations may indicate decay, stains, or merely mold growth.

Cultural methods are used for the isolation of the agents producing decay and for the differentiation of various types. Media suitable for the desired purpose are used. Bavendamm[1] used a 2 per cent malt agar containing 0.2 per cent tannic or gallic acid to differentiate between white-rot fungi and brown-rot fungi. Colonies of the former fungi produced dark halos or oxidation rings on the special agar due to the oxidases that they secreted; while the latter fungi did not produce such diffusion zones. Davidson, Campbell, and Blaisdell,[2] as the result of extensive research, have shown that the generalization of Bavendamm was essentially correct. They used 0.5 per cent concentrations of gallic or tannic acid in malt agar.

By the use of the microscope one may ascertain the presence of mold hyphae, boreholes (made by the perforation of the cell wall by hyphae), corrosion, and spiral cracks in the wood. Hubert's stain is helpful in the foregoing connection.

Hubert's Stain for the Examination of Wood.[3]—Small pieces of wood, about 1 cm. cubed, are boiled in water for 30 min. and then soaked in a solution of equal parts of glycerol and ethanol until they become sufficiently soft to be cut readily with a razor blade.

Thin sections are cut from the samples thus treated and immersed in a 2 per cent Bismarck brown solution (in 70 per cent ethanol) for 1 to 2 min., the time depending on the kind of wood, its density, the thickness of the sections and the degree of deterioration of the wood. The excess of Bismarck brown is drained from the sections, which are then washed with distilled water. The sections are next immersed in a solution of methyl violet (1 part of a saturated aqueous solution of methyl violet mixed with 3 parts of distilled water) for 2 to 5 min. Under certain circumstances it may be desirable to use the saturated solution of the dye, staining for 1 to 2 min. The sections are now washed with distilled water.

The stained sections are mounted in water on a glass slide and examined microscopically. If the violet color appears to be faint, the sections

[1] Bavendamm, W., *Zeit. Pflanzenkrankh. Pflanzenschuty.*, **38**: 257 (1928).

[2] Davidson, R. W., W. A. Campbell, and D. J. Blaisdell, *Jour. Agr. Research*, **57**: 683 (1938).

[3] Hubert, E. E., *Phytopathology*, **12**: 440 (1922); Thaysen, A. C., and H. J. Bunker, "The Microbiology of Cellulose, Hemicelluloses, Pectins and Gums," Oxford University Press, New York, 1927.

should be stained again with methyl violet. If the Bismarck brown is faint, the entire staining procedure should be repeated. Satisfactory sections are covered with a cover glass, to prevent the sections from curling, and dried slowly. (Egg albumen or gum arabic may be used to fix curled sections.) Permanent mounts may be made of the dried sections, using balsam or other material.

In a satisfactory stain, the mold hyphae become deep violet in color; the cell walls of the wood become yellow to brown; the wood tissues with exposed cellulose yield a mixed brown and violet color; and the contents of "medullary rays and the boarded pits of conifers" are dyed to a violet color usually.

BACTERIA

Bacteria and Wood Decay.—Bacteria exert a minor role in the decay of wood. Saprophytic types occasionally may increase the rate of decay by fungi. Under certain abnormal conditions cellulose-decomposing bacteria may bring about some breakdown of the wood. In general, it may be repeated that the higher fungi are mainly responsible for the destruction of wood.

There are several nonmicrobic biological agencies of wood destruction that are of great importance, and although they do not fall within the general scope of this work it may not be out of place to give a brief resumé of them here. For fuller information the reader is referred to works dealing specifically with these animals.

INSECTS

Termites.—Termites are insects that belong largely to the families *Termitidae, Kalotermitidae,* and *Rhinotermitidae.*[1] In the eastern part of the United States, *Reticulitermes flavipes* (Kollar) is a very common species.[2]

Termites occur where the climate is warm, generally in the tropical and temperate regions. They cause great damage to wooden structures, the losses amounting to millions of dollars annually.

Although termites resemble ants in some respects and have been erroneously called "white ants," they are not ants. Their social organization, in some ways, is similar to that of ants. They live in colonies, a single colony sometimes containing thousands of individuals.

Workers (the termites largely responsible for building the colony, securing the food, and providing for the young) make up more than 75

[1] Kofoid, C. A. *et al.*, "Termites and Termite Control," 2d ed. University of California Press, Berkeley, 1934.

[2] Metcalf, C. L., and W. P. Flint, "Destructive and Useful Insects," 2d ed., McGraw-Hill Book Company, Inc., New York, 1939; Snyder, T. E., "Our Enemy the Termite," Comstock Publishing Company, Inc., Ithaca, N.Y., 1935.

per cent of the termites. They are whitish in appearance and do not possess wings. They cannot produce young, for they are sterile.

Termites may be classified either as wood-dwelling or as subterranean termites.[1] The wood-dwelling termites include damp-wood termites and dry-wood termites. The former are found frequently in decaying, moist wood; while the latter are found in wood of relatively low moisture content.

The subterranean termites comprise a large part of the termites. The genera *Coptotermes*, *Reticulitermes*, and *Heterotermes*, of the family *Rhinotermitidae*, are known as "subterranean termites" and cause much

Fig. 56.—Work of the eastern subterranean termite in the sill of a house. Note the concentric arrangement of the passages due to leaving the harder summer wood in each annual ring. The knots are also not attacked. [*Courtesy C. H. Blake, Tech. Rev.*, **41** (*No.* 3) (1939).]

of the destructive action of economic importance. These termites are widespread in distribution and are very devastating in their action. New colonies may start in the ground or on the surface of the ground, damp or partially decayed wood forming a good site. Moisture is required.

When subterranean termites attack the wood of a building, they may construct a covered passage between the colony and the site of their tunneling operations. The soil, wood, fecal matter, and other material they use in building the passage form a plaster that is characteristic.

The initial attacks of termites are usually made on the outer walls of a dwelling near the ground. Such wood may appear to be sound even though injured by termites. By tapping on the wood or by pressing on it, the damaged condition may be ascertained.

The termites cut off small pieces of wood with their jaws.[2] The cellulose present in the wood is broken down to sugars by the Protozoa

[1] Kofoid, *op. cit.*

[2] Blake, C. H., *New England Mus. Nat. Hist., Leaflet* 3, 1937; *Tech. Rev.*, **41** (No. 3), January, 1939.

that are present in the stomachs of the termites. The Protozoa possess enzymes that have the ability to transform the cellulose, thus making the wood available as food for the termites.

Although many termites cause destruction of useful materials, some are useful in returning dead wood to the soil.

The prevention of destruction by termites may be largely achieved by the use of proper construction methods. Where termites are known to be particularly destructive, materials that cannot be attacked should be used. The use of a shield of copper, or some other metal, between the foundation and the structure (if the building is to be made of wood) is advantageous. When timbers may come into contact with the soil, pressure impregnation with creosote is advocated. Creosote oil is good

FIG. 57.—Surface of an oak board infested with powder-post beetles. The exit holes are shown. They have a diameter of about 1/16 in. [*Courtesy C. H. Blake, Tech. Rev.*, **41** (*No.* 3) (1939).]

when discoloration, inflammability, and odor are not considered undesirable. Timbers treated with zinc chloride are clean, odorless, and may be painted. Chromated zinc chloride is said to fix readily with wood and to offer some resistance to fire.

For further information concerning termites and termite control, consult some of the publications listed at the end of this chapter.

Powder-post Beetles.—The powder-post beetles (*Lyctidae*) are the most important of the insects producing the damage known as "powder-post defect." The adult females lay eggs in the wood. The latter develop into larvae, which live principally on starch. Tunnels of an irregular nature are excavated by the grubs. The fine powder resultant from their burrowings may fall out, producing characteristic heaps of wood sawdust. The injury caused by the powder-post beetles is confined to the sapwood of woods of broad-leaved trees such as ash, hickory, oak, and other woods.[1]

[1] *U.S. Dept. Agr., Bull.* 333 (1916); 1232 (1924); and *Farmers' Bull.* 1472 (Revised 1934).

Carpenter Ants.—Carpenter ants are large black ants about 0.25 to 0.5 in. in length. These insects dig tunnels and galleries in wood. The passages made by them are larger, in general, than those produced by termites and contain none of the plaster characteristic of the latter insect. The excavated wood is not used for food, the passages serving merely as shelters. Posts, structural timbers, and occasionally dwellings are attacked. The ants are very common in forests, where they may be found in stumps and in fallen or standing trees that are partially decayed.

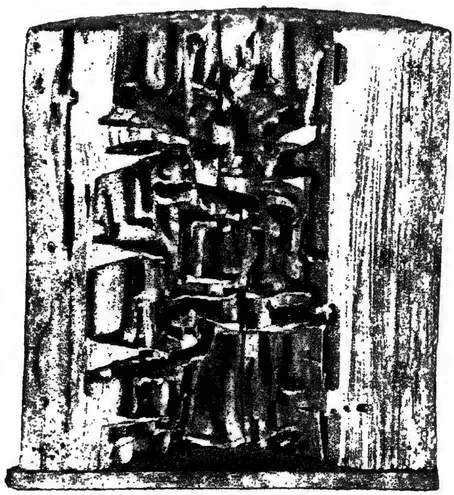

FIG. 58.—Half section of part of a pole worked by carpenter ants. The true height of this specimen is 11 inches. [*Courtesy C. H. Blake, Tech. Rev.*, **41** (*No.* 3) (1939).]

THE MARINE BORERS

The marine borers include molluscan and crustacean borers. The molluscan borers, or shipworms, include the important genera *Teredo*, *Pholas*, *Martesia*, *Xylophaga*, *Lithodomus*, *Zirphaea*, and *Petricola*.[1]

Marine borers cause annually millions of dollars' worth of damage to wharves, docks, yachts, and other marine craft and structures. Of the marine borers, *Teredo* is by far the most destructive.

Teredo.—This borer is found in many parts of the world. In this country it occurs on the Atlantic, Gulf, and Pacific coasts.

The female forms eggs that may be fertilized in its body or in the water, depending on the species. The eggs develop into larvae, which have bivalve shells and are free-swimming. It is during the larval period

[1] CLAPP, W. F., *Civil Eng.*, **7**: 105 (1937).

that the individuals are able to move about. After a few days the larvae swim to a structure or piece of wood and attach themselves. Entrance to the wood is made at right angles to the grain. The tunnel thus made is of small diameter. Later the teredo burrows with the grain, growing at the same time. Consequently the excavations become larger. The teredo, once within the wood, never leaves it, the wood serving as a shelter and as part of its food supply.

The anterior end of the shipworm contains the valves, which are active in boring. The posterior end is supplied with two tubes; one of

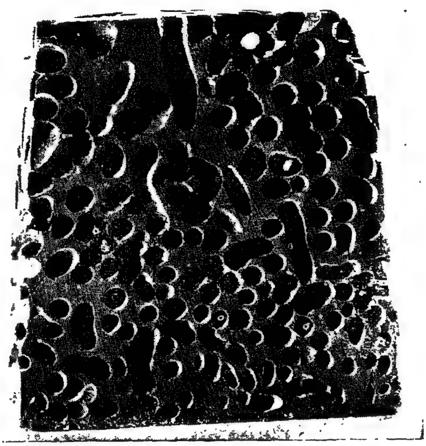

FIG. 59.—Work of teredos. A block cut from damaged wood. Average diameter of the holes is about ¼ inch. Note the white, limy lining. The passages do not intersect. [*Courtesy C. H. Blake, Tech. Rev.*, **41** (*No.* 3) (1939).]

these takes in water (containing dissolved oxygen and food) and the other functions as an excurrent siphon to expel water, wood, wastes, and other substances.

The adult teredo sometimes reaches a length of 1 to 4 ft. and a diameter of nearly 1 in. Wood infested with these organisms will thus weakened, the extent and rate of destruction depending on the numbe present; the species; the food available; the temperature, salinity, pH, and dissolved oxygen of the water; the presence or absence of pollution; and many other considerations.

Clapp[1] reports that *Teredo navalis* has been outnumbered by the larger and more destructive *T. megotara, T. dilatata, T. norvegiae,* and *T. Thompsoni* in New England harbors.

[1] CLAPP, W. F., *Lab. Bull.* 5, Jan. 17, 1938.

Control of marine borers is, of course, very important. Research has done much to aid man in learning of the habits of the borers. Test boards have been suspended at various points in harbors and along the coast to determine the species and numbers present and the conditions favoring or inhibiting their presence. Certain test boards have been treated in various ways with chemical agents or by other means to discover methods of resisting the attack of the borers.

Studies of associated organisms have yielded much information of value that may be used in predicting whether a certain location may at some future time be subject to the attacks of marine borers.

Timbers and structures may be treated in various ways to resist attack. Wood impregnated with creosote is resistant. The protection of timbers by metal and masonry where they are exposed to water is of great value.

Boats may be protected by the application of special marine paints. According to Clapp, the timber keels, the yarboard planking, the shaft logs, and the centerboard wells are most susceptible to attack. It is important to paint all exposed surfaces, for the borers may tunnel in on submerged portions where the boat is unprotected by paint.

Martesia.—The individuals look much like small clams and may grow to a length of 2.5 in. and a width of 1 in. Much damage to marine structures is caused by this borer, principally on the Gulf of Mexico in this country.

The young of this genus move about in the water without restriction. They bore into timbers when small, the entrances being usually ⅛ in. or less[1] in diameter.

Crustacean Borers. *Limnoria lignorum.*—The destructive crustacean borers of importance include the isopods *Limnoria* and *Sphaeroma*, and the amphipod, *Chelura terebrans.* The most serious damage to wood caused by crustacean borers is brought about by *Limnoria lignorum*, known also as "gribble." *Limnoria* has been found in many parts of the world in both warm and very cold waters. Most of the species are not associated with economic loss. The adult is not large, seldom exceeding 0.2 in. in length.[2]

Under favorable conditions, adults or half-grown individuals (never the young) may tunnel into piles or other marine timber, some individuals boring as far as 20 mm. They may attack the wood at various levels. Sometimes they confine their attacks to points 1 to 2 ft. above and below the mean water level; at other times they may attack at the mud line at points as much as 70 ft. below the mean low water level.

[1] Hunt and Garratt, *op. cit.*

[2] Clapp, W. F., The Boring Crustacea of the North Atlantic, *Wm. F. Clapp Laboratories, Rpt.* 361, Dec. 1, 1935.

The female produces a small number of eggs, usually less than 20, which hatch after an incubation period of 2 to 6 weeks. The young burrow from the tunnel of the mother, usually seeking the softer portions of the wood. Very small holes are bored from the burrows to the outside, which facilitate the entrance of water and removal of waste materials. A piece of wood subjected to a heavy attack by *Limnoria* presents a characteristic lace-like appearance.

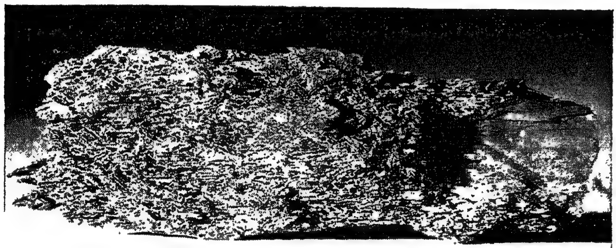

FIG. 60.—Gribble work. This specimen is about ½ in. thick with a sound back surface. The holes are nearly parallel to the outer surface and about 1/16 inch in diameter. [*Courtesy C. H. Blake, Tech. Rev.*, **41** (*No.* 3) (1939).]

As much as 0.5 in. of outside wood may be destroyed during one year. The wood thus injured may be broken off by the action of the waves or floating objects, exposing a new surface into which specimens soon bore.

Specimens of *Limnoria lignorum* may leave their tunnels quickly and easily when the occasion demands, choosing a new location. The entrance to the burrow of an individual may be effectively blocked by its telson (flattened tail) when the specimen becomes alarmed.

Although hardwoods may be injured to some extent by *Limnoria*, the borers having mandibles capable of cutting through the hardest woods, these crustacea are usually found in softwoods.

The degree of salinity is an important factor in the life of *Limnoria*. When the salinity is reduced to 5 parts per 1,000, the borer is usually destroyed in less than 1 hr. A concentration of only 20 parts per 1,000 is inhibiting. Hence heavy rains, or other factors producing a dilution of the water, may cause a rapid destruction of *Limnoria* colonies in a harbor.

Heavy silt is an important agent in inhibiting the action of *Limnoria lignorum*, for it tends to block the passage of water through the tunnels.

Sphaeroma.—An individual of this genus in many respects resembles a large *Limnoria*. Although destructive, it is of much less importance than *Limnoria*.

Chelura.—*Chelura terebrans*, a species of amphipod, is said to be a cause of much destruction, particularly in European waters. This

species, according to Clapp, has displaced *Limnoria* from certain localities of the North Atlantic Coast.

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APPENDIX A

DETERGENCY, DISINFECTION, AND STERILIZATION

The industrial microbiologist encounters other problems than those dealing with the cultivation and large-scale propagation of the organisms he is to employ as biochemical reagents for the transformation of organic materials. These problems involve the cleanliness and care of the equipment or apparatus he is to use, and the knowledge of how it can be made and kept free from invading microbes. Information on the general principles of detergency, disinfection, and sterilization will be found useful even though they have been worked out particularly in processes pertaining to public health and general sanitation.

Every bacteriologist recognizes the importance of employing sterile media and apparatus. Similarly, success in all technical fermentations depends on the application of some method or methods for inhibiting or destroying undesirable microorganisms, which are the ever-present enemies of the industrial microbiologist. The method used depends on the nature of the material to be sterilized and on the processes that may be affected. Where heat may be produced and applied cheaply without injury to the chemical character of the ingredients, steam is an excellent agent and the most reliable one to use for sterilizing mashes, fermentable solutions, etc., and for treating vats, pans, pipe lines, and other equipment. In certain industries, such as the acetone-butanol industry, where asepsis is so very important, steam, usually under pressure, is used in preference to all other agents. Other methods of sterilization, either physical or chemical, may be employed in individual cases, as in the sterilization of walls, floors, bench tops, etc. Before discussing these particular aspects of the subject further, it is well to define and explain the meanings of some of the terms used in connection with the destruction or inhibition of microbial life. Following this a brief enumeration of the types of agents available for this kind of work will be given.

Sterilization literally signifies the destruction of all living cells in a medium or environment. In commercial work the word is sometimes loosely employed to indicate a process of heating with the intent to kill germs. Heat is the most common sterilizing agent.

A *germicide* is anything, but especially a physical or chemical agent, that destroys germs. Patterson[1] states that "in practice it is assumed that a substance represented as a germicide, when used as directed, will kill all ordinary disease germs, but is not necessarily required to be capable of destroying bacterial spores." He further states that "in combatting diseases (such as anthrax or tetanus) caused by spore-forming bacteria, germicides or procedures specially effective against spores will be required." Obviously in technical fermentations the term "vegetative cells" should be substituted for "disease germs."

A "*bactericide* is anything that destroys bacteria."[1] It does not necessarily destroy the spores of bacteria, however. Furthermore it is a more restricted term than germicide.

A *fungicide* is anything that destroys fungi.

[1] Patterson, A. M., *Am. Jour. Pub. Health*, **22**; 465 (1932).

An *antiseptic* is a "substance that opposes sepsis, putrefaction or decay; one that prevents or arrests the growth or action of micro-organisms, either by inhibiting their activity or destroying them; used especially of agents applied to living tissues."[1]

Bacteriostasis is a term signifying a suspension of animation and reproduction by bacteria due to the influence of an agent known as a "bacteriostatic agent." It does not imply destruction of bacteria, although bacteriostatic agents, in high concentration or acting over a long period of time, may permanently destroy the ability of the bacteria to reproduce. The term "bacteriostasis" was introduced by Churchman in 1912. Some of the triphenylmethane dyes, such as crystal violet and brilliant green, and mercurials possess high bacteriostatic properties, especially in relation to certain Gram-positive bacteria.

Fungistasis, similarly, signifies a cessation of animation and reproduction by fungi.

A *disinfectant* is an "agent that frees from infection; usually, a chemical agent which destroys disease germs or other harmful micro-organisms (but not, ordinarily, bacterial spores); commonly used of substances applied to inanimate objects."[1]

Disinfection and sterilization will be discussed in this chapter primarily in relation to their industrial aspects.

Agents.—Agents for sterilization include physical and chemical agents. Physical agents include the use of high temperatures applied either as steam or dry heat for sterilization and pasteurization; the use of sunlight and ultraviolet light; the use of drying, pressure, high-frequency sound waves, or electrical currents; and filtration methods. Chemical agents include a very large list of substances from simple salts or soaps to special compounds, some of which are of complex structure. For the sake of simplicity, chemical agents may be classified on the basis of their chemical structure into acids; alkalies; phenols and phenol derivatives; salts and compounds of heavy metals; alcohols; halogens; dyes; and other compounds. In Table 95 several examples from the great number of the various classes of chemical agents that may be employed in disinfection with good results are given, although the agents mentioned are not necessarily better than many others unlisted.

TABLE 95.—SOME CHEMICAL AGENTS USED AS ANTISEPTICS, GERMICIDES, OR DISINFECTANTS

Acids	Alcohols	Alkalies	Halogens	Heavy metals and compounds of them	Phenols and related compounds	Dyes	Miscellaneous
Acetic	Butyl (tertiary)	Sodium carbonate	Chloramines:	Mercuric chloride	Alkyldimethylammonium chlorides (zephirol)	Acridine	Alkyl sulphates
Benzoic	Ethyl	Sodium hydroxide	Azochloramid	Mercurochrome (in alcohol)	Alkylaryl sulphonate	Proflavine	Formaldehyde
Boric		Trisodium phosphate	Chloramine-T	Mercury oxycyanide	2-Chloro-*o*-phenylphenol	Triphenylmethane:	Organic peroxides
Formic			Dichloramine-T	Merthiolate	Creosotes	Brilliant green	
Lactic			Chlorine	Metaphen	Hexylresorcinol	Crystal violet	
Salicylic			Hypochlorites:	*o*-Hydroxyphenylmercuric chloride	Pentachlorophenol	Gentian Violet	
Sulphurous			Calcium hypochlorite	Phenylmercuric nitrate	Phenol		
			Sodium hypochlorite	Silver (colloidal)	*o*-Phenylphenol		
			Fluorides	Silver compounds	Tetrachlorophenol		
			Iodine		Tricresols		
			Iodine compounds				

[1] *Ibid.*

The Phenol-coefficient Test.—This test is used in some form in nearly every civilized country in the world as the procedure for comparative rating of disinfectants. It is a "standard" procedure used by the Food and Drug Administration,[1] which annually examines hundreds of samples of disinfectants, germicides, and bactericides. Provided that a variety of conditions and a fairly large number of test organisms are used, this test may supply much valuable information. On the other hand it may require only a few tests to indicate that a substance has no particular value as a disinfectant.

Limitations of the Phenol-coefficient Test.—The phenol-coefficient test was designed to evaluate disinfectants that were closely related to phenol in chemical structure. This test is unsuited for the evaluation of substances quite unlike phenol and for substances insoluble in water. It gives no information concerning the ability of the chemical agent to penetrate organic matter, nor its toxicity to tissues.

Definition of Phenol Coefficient.—The phenol coefficient is a term that expresses the germicidal action of a chemical agent toward a test organism at a given temperature in terms of the action of phenol under identical conditions. Thus a phenol coefficient of 5.0 means that an agent is five times as effective as phenol under a given set of conditions, which should be specified. Should a phenol coefficient be stated on the label of a container without the name of the test organism or the temperature of the test, then it is understood that the test was carried out with *Eberthella typhosa* (Hopkins strain) at a medication temperature of 20°C.

E. typhosa (Hopkins strain), which may be secured from the Food and Drug Administration at Washington, D.C., is the standard test organism used in testing disinfectants. This organism is cultivated under a standard set of conditions, as outlined in *Circular* 198, *Food and Drug Administration, U.S. Department of Agriculture,* and should demonstrate a fairly constant resistance to phenol at 20° and 37°C. Should its resistance vary appreciably, a new subculture should be obtained for test purposes.

Calculation of the Phenol Coefficient.—To calculate the phenol coefficient, the denominator of the fraction expressing the highest dilution of the chemical agent that destroys the test organism in 10 but not 5 min. is divided by the denominator of the fraction expressing the highest dilution of phenol that destroys the test organism in 10 but not 5 min. under the same set of conditions.

Let us suppose that a test was carried out according to Food and Drug Administration methods, using *Staphylococcus aureus*, Government 209, a special strain that may be used in testing germicides and antiseptics, as the test organism at a medication temperature of 20°C., and let us suppose that the data shown in the table on page 494 were obtained.

The phenol coefficient, calculated from these data, is $^{150}/_{60} = 2.5$.

Procedure in the Phenol-coefficient Test.—The Food and Drug Administration test is made in the following manner. Dilutions of phenol and of the disinfectant, or germicide, are prepared in a series of sterilized medication tubes (Pyrex tubes with a diameter of 25 mm. and a height of 150 mm.). Just 5 cc. of the chemical agents or of dilutions of them are left in each tube. The tubes are placed in a rack in a water bath adjusted to the desired temperature and permitted to stand for at least 5 min. before the test is carried out, in order to bring the temperature of the contents of the tubes to that of the bath. The bath should be provided with an efficient stirrer and an accurate thermostat capable of maintaining the temperature within 0.1°C. or less, of that desired.

The test culture, which should be 24 hr. old (22 to 26 hr.) and which should be subcultured successively at daily intervals for at least 5 days before use, in order to

[1] *U.S. Dept. Agr.*, F.D.A., *Circ.* 198, December, 1931.

activate it, is shaken vigorously to break up the small clumps of bacteria that may be present and is placed in the water bath at least 15 min. before the test is carried out. Sufficient of the test culture is drawn into a single sterile pipette to inoculate every tube of the series. To each 5 cc. portion of the chemical solutions in the medication tubes is added 0.5 cc. of a broth culture of the test organism. At intervals of 5, 10, and 15 min., a 4-mm. loopful (No. 23 B. & S. gauge platinum wire) of material is withdrawn from each tube and planted in a corresponding tube of Reddish broth, or of the medium that is especially adapted for the growth of the organism being studied. The subculture tubes are incubated at the temperature most favorable for the growth of the test organism. Preliminary observations may be made after 24 hr., but final observations should not be made until the end of 48 hr. Under certain conditions, it may be advisable to incubate the subculture tubes for longer periods of time, especially if a substance suspected of possessing high bacteriostatic properties is being examined.

Chemical agent	Dilution	Time intervals, minutes		
		5	10	15
Phenol	1/60	+	0	0
Phenol	1/70	+	+	+
Disinfectant	1/120	0	0	0
Disinfectant	1/130	0	0	0
Disinfectant	1/140	+	0	0
Disinfectant	1/150	+	0	0
Disinfectant	1/160	+	+	+
Disinfectant	1/170	+	+	+
Disinfectant	1/180	+	+	+
Disinfectant	1/190	+	+	+

NOTE.—A + sign signifies that *Staphylococcus aureus* was not destroyed by the chemical agent at the time interval indicated, while a 0 sign indicates that it was destroyed.

In the case of mercurials or other agents possessing high bacteriostatic properties, it is essential to make secondary subcultures from the subculture tubes immediately after the test has been completed. This may be accomplished by transferring one to four 4-mm. loopfuls of material from each subculture tube to corresponding tubes of sterile culture medium. Secondary subculture tubes should be incubated along with the primary subculture tubes. Growth in the secondary tubes but not in the primary tubes is a reliable indication of the bacteriostatic nature of the chemical agent being examined. Other indications of bacteriostasis are the appearance of growth in primary subculture tubes later than 48 hr., or the appearance of "skips" or of irregularities in the data secured from the 48-hr. observation.

Occasionally a chemical substance may be added to the subculture medium, which neutralizes any bacteriostatic effects. For example, hydrogen sulphide may be added in the case of mercuric chloride, or sodium thiosulphate, in the case of hypochlorites.

The phenol coefficient is calculated from the data of the experiment, provided they are adequate. It is always desirable to duplicate results. Estimated values should be substantiated by further tests.

It is to be repeated that substances quite unlike phenol should not be examined by this method but by others that are more appropriate.

For other details concerning this test the student is referred to *Circular* 198, *Food and Drug Administration, U.S. Department of Agriculture* (December, 1931).

Since the publication of the U.S. Food and Drug Administration Methods of Testing Disinfectants and Antiseptics, several methods for evaluating germicides and antiseptics have been reported. Although these are not now to be regarded as standard, they offer, in many instances, substantial improvements in methods of evaluation. They may be regarded as other types of measuring sticks.

Table 96 summarizes some information concerning some of the more recent tests.

TABLE 96.—SOME RECENT METHODS FOR EVALUATING GERMICIDES AND ANTISEPTICS

Nature of method	Special feature	Authors	References
Determination of toxicity to tissues; tissue-culture technique	Toxicity index	Salle, McOmie, Shechmeister, and Foord	*Proc. Soc. Exptl. Biol. Med.*, **37**: 694 (1938); etc.
Toxicity and germicidal action measured by manometric method	Toxicity index. Inhibition of oxygen consumption by tissues and bacteria, due to germicide, measured	Bronfenbrenner, Hershey, and Doubly	*Proc. Soc. Exptl. Biol. Med.*, **38**: 210 (1938); *Jour. Bact.*, **36**: 265 (1938); **37**: 583 (1939)
Toxicity to leucocytes	Phagocytic action after treatment with germicide measured	Nye	*Jour. Am. Med. Assoc.*, **108**: 280 (1937)
Method for studying toxicity	Goldfish used as test animals	Gersdorff	*Jour. Am. Chem. Soc.*, **52**: 3440 (1930)
Therapeutic value	Abdomens of albino mice inoculated with invasive strains of *Staphylococcus aureus* and germicide	Hunt	*Jour. Infectious Diseases*, **60**: 232 (1937)
Determination of irritation to skin	Stoppered glass rings strapped to skin of animal	Etchells and Fabian	*Jour. Ind. Hyg.*, **17**: 298 (1935)
Germicidal action of chlorine compounds	Spores of *B. metiens* used. Chlorine neutralized by $Na_2S_2O_3$ and samples plated	Charlton and Levine	*Iowa State Coll. Agr. Mech. Arts, Eng. Expt. Sta., Bull.* 132, 1937
Fungicidal test	Molds cultivated in petri dishes; molded agar cut into squares, fungicide added, squares rinsed and subcultured	Burlingame and Reddish	*Jour. Lab. Clin. Med.*, **24**: 765 (1939)
Evaluation of germicides for treating surgical instruments	Instruments contaminated with sporeformers, etc.; germicide applied; survivors counted	Reddish and Burlingame	*Jour. Bact.*, **36**: 265 (1938)
Testing antiseptic dusting powders	Pasteboard shields used to limit field of application in seeded agar in petri dishes	Brewer	*Jour. Bact.*, **37**: 411 (1939)
Method for testing non-phenolic disinfectants	Varnished sticks (8 by 4 by 100 mm.) flamed, impregnated with *Staphylococcus aureus*, dried, treated with disinfectant, placed in tubes of sterile broth	Clark	*Jour. Am. Pharm. Assoc.*, **27**: 130 (1938)
Phenol coefficients of relatively insoluble compounds	Sulphonated oil causes uniform dispersion of germicide in water	Carswell and Doubly	*Ind. Eng. Chem.*, **28**: 1276(1936)

Requirements of a Disinfectant.—The requirements of a disinfectant will depend on the purposes for which it is to be used. In general, a disinfectant should possess high germicidal activity; should be effective in the presence of organic matter and at the pH and temperature used; should be stable; should be water soluble (for many purposes); should impart no undesirable color or odor; should possess no undue toxicity for animals; and should be capable of being produced at a reasonable cost. Obviously it is not an easy matter to produce a chemical agent that is ideal in all ways.

A chemical agent would have little value if it did not possess the ability to destroy various types of bacteria, molds, and/or yeasts, pathogenic and nonpathogenic, under the conditions of use. Since most substances of this nature are ineffective or merely antiseptic in very dilute form, the disinfectant must not be used in too low a concentration if it is to be effective. A useful method for calculating a satisfactory dilution is to multiply the so-called "phenol coefficient" (a figure obtained under special conditions at 20°C. using *Eberthella typhosa* (Hopkins strain) as the test organism) by 20. The reciprocal of this figure is the dilution that may be used. For example, a disinfectant with an *E. typhosa* phenol coefficient of 5 might be employed in a dilution equal to the reciprocal of 20 × 5, or $\frac{1}{100}$, that is, 1 part of disinfectant in 99 parts of water. This is the concentration that corresponds to a $\frac{1}{20}$, or 5 per cent, phenol solution.

Almost all disinfectants are reduced in chemical efficacy by the presence of organic matter. This is true of hypochlorites, mercurials, and other compounds. Phenol and tricresols are not so much affected by organic matter as certain other disinfectants.

The reaction at which a disinfectant is used is at times very important. Hypochlorites are very much more effective at a slightly acid reaction. Certain other compounds are more efficacious when the pH is relatively high. The flavines, for example, acriflavine, are more effective when the reaction is alkaline.

Temperature is a very important factor in disinfection. It is a well-known fact that chemical reactions usually proceed more rapidly as the temperature is elevated. Disinfectants, as a rule, are more effective at 37°C. than at 20°C. or at lower temperatures. There are apparent exceptions to this rule, however.

It is important to have information concerning the solubility of the disinfectant in water and other solvents. For most practical applications of disinfection, water-soluble types of chemical compounds are essential, as, for example, in treating a water supply or in sterilizing the walls of tanks, pipes, etc. Under certain conditions it may be desirable to impregnate substances, such as fish nets, tents, or tarpaulins with a substance that will not be washed out when the fabric comes into contact with water, as it must sooner or later.

Stability is an important requirement for a disinfectant. Most chemicals are not used at once after their manufacture. They may remain for months as unsold stock. Even after purchase by the ultimate consumer, the agent may not be used immediately. It is, therefore, important that the disinfectant should be stable or that the approximate rate of its decomposition be known. Cognizance is taken of these facts in the distribution of liquid hypochlorites. For this type of disinfectant, it is important that the date of manufacture, the quantity of available chlorine at that date, expressed in percentage, and the approximate rate of decomposition when stored in a cool, dark place at a temperature not higher than 20°C. be known.

Ways and means for aiding in the stabilization of relatively unstable compounds are known in many cases. Hypochlorites may be partially stabilized by adjusting the pH to extreme alkalinity through the use of sodium and/or calcium hydrates, sodium carbonate, or other suitable alkaline substances. Obviously the pH of these compounds must be adjusted to a much lower value for effective germicidal action.

This may be done at the time of use. Peroxides may be stabilized by the use of small amounts of acetphenetidide or of acetanilide or by other means. For example, Perhexogen is a hydrogen peroxide in a solvent of tertiary butanol with a trace of metaphosphoric acid as a preservative.[1]

Under some circumstances it may make no special difference whether a disinfectant imparts a color or not, but usually one that does not add color is desirable. Creosotes are excellent disinfectants for the treatment of wood, but they cannot be used on surfaces that are to be painted, or the inner surfaces of vats or tanks.

The question of odor is an important one also. Phenols and cresols cannot be used in food establishments near foods on account of the readiness with which many types of foods absorb odors. One part of phenol in several million parts of water may be readily detected by the average individual, and its odor may be imparted to foods even when the dilution is great. Compounds must not be used to treat interior woodwork if they evolve undesirable odors.

Disinfectants intended for general plant purposes where there is likely to be contact with the hands or skin must not possess too great toxicity. In the disinfection of stables, it is essential to use a disinfectant which will destroy pathogens but which will not injure livestock. Certainly disinfectants used in the treatment of equipment that is used in connection with manufacture of food or beverages must not possess high toxicity. Chlorine compounds, chlorine, and hypochlorites are especially valuable for the latter purposes. Scales[2] has recently pointed out the possibilities of using alkyl aryl sulphonate, either alone or with a chlorine solution. Spores were readily destroyed by low concentrations of the compound.

The question of toxicity to tissues is, of course, of paramount importance in the case of germicides and antiseptics.

Chlorine Solutions.—Chlorine solutions are of three general types: aqueous solutions of chlorine, hypochlorite solutions, and chloramine solutions. Chlorine is much used in the treatment of water supplies, swimming pools, shell-fish, water used for cleaning fish, and for many other purposes. Hypochlorites include sodium hypochlorite ($NaOCl$), which has been made alkaline with sodium hydroxide, calcium hydroxide, sodium carbonate, or some other alkaline salt; and calcium hypochlorite, which has been made alkaline with calcium hydroxide. Dakin's solution is a hypochlorite in which boric acid is used to reduce the alkalinity to a pH of slightly greater than 7. Zonite, Bacili Kil, Eusol, and Perchloron are hypochlorite solutions.

The chloramine compounds include chloramine-T (*p*-toluene sodium sulphonchloramide), dichloramine-T (*p*-toluene sulphon-dichloramide), and Azochloramid (*N,N′*-dichloroazo-dicarbonamidine). Chloramine-T is water soluble and liberates hypochlorous acid in aqueous solution. Dichloramine-T is insoluble in water; it is mixed with a chlorinated oil. Azochloramid liberates chlorine very slowly and is said to produce good results in surgical antisepsis.

A commercial standard for "liquid hypochlorite disinfectant, deodorant and germicide" (CS 68-38) became effective on June 10, 1938.[3] Herein it is stated that the chlorine content of a hypochlorite solution shall appear on the label and shall not be less than 2.5 per cent. When stored in the original container in a dark place at a maximum temperature of 20°C. (68°F.), the rate of deterioration of the compound shall not be greater than 10 per cent of the original content of available chlorine in 6 months.

[1] COMBES, F. C., *N.Y. State Jour. Med.*, Nov. 15, 1937.

[2] SCALES, F. M., paper presented to Laboratory Section of International Association of Milk Dealers, Oct. 17, 1938.

[3] *U.S. Dept. Comm., Nat. Bur. Standards, Com. Standard,* CS68–38, 1938.

Considerable research concerning chlorine solutions has been carried out. Significant papers have been presented by Johns, Charlton and Levine, Costigan, and others. As is the case with many disinfectants, temperature, pH, and concentration are very important factors in connection with the efficiency of germicidal action. Hypochlorites and chloramine-T solutions are strongly affected by pH changes, being very much more effective germicidally in slightly acid solutions than in alkaline solutions. Chloramine solutions appear to be less affected by pH changes.[1]

Charlton and Levine are of the opinion that the undissociated hypochlorous acid (HOCl) is the most important germicidal agent of hypochlorites. Undissociated hypochlorous acid is apparently not the significant factor in the case of simple chloramines or chloramine-T, where it is believed that the positively charged chlorine atom is mainly responsible for the disinfecting action.

In general, the germicidal efficiency of chlorine and hypochlorites is considerably reduced by the presence of organic matter. Accordingly, it is essential to clean a surface thoroughly before applying the chlorine solution.

Detergents.—A detergent is a substance that cleanses.

The more common detergents include caustic soda (NaOH), soda ash or sodium carbonate (Na_2CO_3), trisodium phosphate (Na_3PO_4), sodium metasilicate, and sodium hexametaphosphate [$(NaPO_3)_6$ or $Na_2(Na_4P_6O_{18})$]. Various mixtures of the foregoing are employed.

Caustic soda forms the base of several cleaning solutions. The concentration of sodium hydroxide used will depend upon the type of work being done. The American Bottlers of Carbonated Beverages state that a minimum of 3 per cent alkali solution should be used in treating unclean bottles, of which not less than 60 per cent (1.8 per cent) must be caustic soda. The bottles must be exposed to this solution for not less than 5 min. at a temperature of not less than 130°C. An "equivalent cleansing and sterilizing process" may be substituted for the foregoing. The usual concentration of sodium hydroxide used may vary from 0.5 to 4 per cent. Many bacterial spores and other resistant bacteria are destroyed under these conditions. For example, Arnold and Levine have reported that *Staphylococcus aureus* was destroyed in 10 min.[2] at 98.6°F. by a 1.5 per cent concentration of caustic soda.

Trisodium phosphate is much used as a cleaning compound. It is less efficient as a germicide than caustic soda. Trisodium phosphate, soda ash, sodium metasilicate, and sodium hexametaphosphate are used in combination with caustic soda to produce more efficient cleansing. Caustic soda produces good deflocculation and emulsification but rather poor wetting and rinsing results. Trisodium phosphate and sodium metasilicate when mixed with caustic soda improve the efficiency of the combination, for these compounds are good rinsing agents, in addition to their other qualifications. Sodium hexametaphosphate aids in the removal of bacteria from glassware.

In any washing operation, the efficiency of the detergents will depend on the thoroughness with which the process is carried out mechanically; the nature and amount of the soil; the kinds and quantities of the detergents used; the ability of the detergents to emulsify and saponify the fatty components of the soil; the solution, wetting, and rinsing abilities of the detergents; the temperature of the cleansing water; the germicidal action; and other factors.

[1] Charlton, D., and M. Levine, *Iowa State Coll. Agr. Mech. Arts, Eng. Expt. Sta., Bull.* 132, 1937.

[2] Arnold, C. R., and M. Levine, American Bottlers of Carbonated Beverages, Washington, D.C., 1938.

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APPENDIX B

THE TREATMENT AND DISPOSAL OF INDUSTRIAL MICROBIOLOGICAL WASTES

Fermentation methods are of great service in the treatment of industrial wastes or of domestic sewage when the amount and character of the organic matter is such as to serve as a substrate for bacterial or other microbic action. Such fermentations are sometimes based on an effort to utilize as fully as possible the fermentable material with the production of some gaseous, or other, product such as methane, which might be utilized as a source of heat energy in the industrial operation of the plant. In other instances the aim is to degrade the organic matter by fermentations so that it is no longer putrescible, or so that the amount of putrescible matter eventually discharged into sewers, streams, or other bodies of water will be reduced to a minimum and thus produce little or no effect on the fish or other natural inhabitants of the waters receiving such partially purified wastes.

The disposal of wastes from industrial plants presents special problems in some localities and in particular types of industries on account of the relatively large content of organic matter and the high biochemical oxygen demand (B.O.D.) of these wastes. Indeed the extra load placed on sewage treatment plants by some of these industrial plants may be the equivalent of the load from the inhabitants of a good-sized city.

Wastes from plants utilizing raw materials rich in carbohydrates, proteins, or fats offer special problems in purification by microbic methods. Thus the wastes from the manufacture of corn sugar or starch; from sugar mills; from milk, butter, and cheese plants; or from canneries and slaughtering and meat-packing establishments may cause serious nuisances and high pollution of the waters into which the sewage is discharged unless there is a high degree of breakdown of organic matter previous to such discharge.

Water is used for many purposes in an industrial plant,[1] the most important of which is in connection with some aspect of processing of materials. Process water, which of course varies considerably in amounts, according to the industry, contains a relatively large amount of organic matter. This may be dissolved, colloidal, or in fine particles that settle on standing. For example, process liquors may contain from 1 to 7 per cent of total solids. The inorganic matter and settleable solids account for less than 50 per cent of the total solids, while much of the organic matter is in true solution and is not removed by the ordinary processes of coagulation. Likewise, ultrafiltration does not remove the matter in true solution, whereas it may reduce the B.O.D. of domestic sewage to zero. Thus industrial sewage differs from domestic sewage in several respects.

Liquid wastes, whether treated or not, are generally disposed of ultimately by the method of dilution, in which they are discharged into streams of fresh water or into large bodies of water, such as the Great Lakes, or tidal waters such as bays. In a small percentage of cases land treatment may be used.

Provided that they have been stabilized or sufficiently diluted, after screening to remove large floating objects, or filtered to take out suspended matter, no nuisances

[1] Buswell, A. M., *Water Works and Sewerage*, April, 1935.

or destructive action will generally arise as the result of the disposal of wastes from fermentation plants. But should there be a lack of dissolved oxygen in the streams as a result of improper treatment of the wastes prior to the disposal of them, nuisances may arise and the water may be rendered incapable of supporting fish and other desirable forms of life.

In general, two methods, one aerobic, the other anaerobic, may be used to stabilize sewage, although various modifications and combinations of the two may be used. In the aerobic method, in which the effluent is of low color and B.O.D., the organic compounds are decomposed with the formation of carbon dioxide, water, and a small residue consisting largely of colloidal and suspended matter. Among the recommended aerobic methods are trickling filters and activated-sludge treatments. In the anaerobic method, methane, carbon dioxide, and a humus-like solid are the main products formed, according to Buswell.[1] The ratio of methane to carbon dioxide varies generally from 1:1 to 3:1, depending on the nature of the wastes and the conditions of the fermentation. A wide variety of organic waste materials, with the exception of mineral oils, may be fermented to produce these gases. The methane evolved may be used as a fuel, while the carbon dioxide might be recovered if in sufficient quantity and used in the manufacture of dry ice or for some other purpose. When conditions permit, an anaerobic method in which the methane formed is used for fuel may be the most economical one for the treatment of trade wastes from large plants. Although there is a 75 to 90 per cent reduction[2] in the B.O.D., this, as well as the color, is still relatively high after anaerobic treatment. Therefore, such effluents are improved by aerobic treatment.

The control of acidity is important in the treatment of industrial wastes, for example, of beer slop from distilleries and of brewery steep water. Wastes that are highly acid cannot be treated directly by aerobic methods, even when the load is small. However, such wastes when neutralized or diluted from 1:10 to 1:100 with water or domestic sewage may be suitably treated. The limit of acidity in the anaerobic treatment when undisturbed continuous fermentation is desired is approximately 2,000 p.p.m., calculated as acetic acid.[2]

The wastes are recovered in the greater number of the large industrial plants, but if the liquid wastes are too dilute, recovery of products is impracticable, and they may then be stabilized by treatment at the plant or by the city treatment plants.

Some Present Methods of Treating and Disposing of Wastes.—Wastes may be disposed of or utilized in several different ways. They may be disposed of by dilution without any preliminary treatment, other than screening. This is done in the case of some plants situated in localities where the sewage is conveyed out to sea or dumped into other bodies of water. Wastes may be anaerobically decomposed and the methane produced used for fuel. The wastes from certain fermentation plants and food manufacturing plants may be concentrated and used as a food for livestock. Certain wastes may be concentrated and, on account of their potassium content, used as a constituent of fertilizers. In rum and whisky plants, from 15 to 35 per cent of the liquid residue from distillation (the distillation slops) is generally used in addition to a fresh supply of water in preparing new mashes. Wastes from small plants, which do not operate continuously, may be disposed of by broad irrigation or lagooning. Other methods for treating wastes are being investigated.

Wastes from Industrial-alcohol Plants.—In an industrial-alcohol plant that uses molasses as the raw material, the slops may have a total solid content of 5 per cent,[3]

[1] Buswell, A. M., *Ind. Eng. Chem.*, **31**: 1349–1351 (1939).

[3] Boruff, C. S., *Ind. Eng. Chem.*, **31**: 1335–1337 (1939).

organic solids amounting to 4 per cent, and a 5-day B.O.D. of about 22,000 p.p.m.—a high pollution load. Such wastes may be concentrated in multiple-effect evaporators and then incinerated. The ash, which contains approximately 33 per cent potassium oxide, is sold to fertilizer manufacturers. When the price for potash is high, the recovery of this slop is worth while. Otherwise recovery adds to the cost of producing alcohol.

Some concentrated slop is dried and marketed as chicken feed. Such material is laxative in nature and hygroscopic.

Such wastes may be digested anaerobically and stabilized aerobically.

Wastes from Yeast Plants.—The wastes from a yeast plant ordinarily contain 1 to 3 per cent of total solids[1] and have a 5-day B.O.D. of 7,000 to 14,000 p.p.m. This waste is too weak to justify the expense of recovery for stock feed, but, on the other hand, the load is sufficiently high to cause serious pollution of streams. Such wastes may be digested anaerobically and then stabilized aerobically by treatment on trickling filters.

Wastes from Breweries.—The liquid wastes from a brewery include those from the brewer's grain, from the recovery of yeast, and from the wash water of the various departments of the brewery. The waste from brewer's grain may contain 3 per cent solids and have a 5-day B.O.D. of 10,000 to 25,000 p.p.m. It has been estimated that the combined wastes from a brewery, per 31-gal. barrel of beer, are equivalent to those of a population load of 15 to 25 persons.[1] Such wastes, however, are too dilute to warrant recovering them as stock feed. They may be disposed of either separately or together with domestic sewage through the usual standard methods of treatment.

Wastes from Distilleries.—The slops from the stills of a distillery may contain from 4.75 to 6 per cent of total solids[1] and 2.5 to 3 per cent of soluble solids and may have a 5-day B.O.D. of 15,000 to 20,000 p.p.m. The slop and wash water from each bushel of grain ground usually amounts to 45 to 55 gal. The solids remaining after the distillation, known as "distiller's grains," are sold as stock feed.

In one large distillery, the liquid wastes are screened, the suspended solids are removed by centrifuges, and the liquor is then evaporated in multiple-effect evaporators. The material thus recovered is sold as a feed.

In large plants in general, the wastes are screened and the screenings dried. The slop may be evaporated, dried, and incorporated with the dried screenings. The combined recovered product amounts to about 18 lb. per bushel of the originally ground grain.[1]

In small distilleries, on the other hand, the slop may be screened, the screenings pressed and dried, and the weak slop disposed of along with domestic sewage. Usually 8 to 10 pounds[1] of dried material are recovered from each bushel of grain ground.

The wastes from a distillery, which contain 3 to 4 per cent of total solids and 0.2 per cent of organic acids, and are still hot, may be fermented by thermophilic bacteria with the production of a mixture of methane and carbon dioxide, at low cost. From 58 to 72 per cent of the organic matter is gasified in 2 to 6 days. The residual sludge is inoffensive and stable, while the liquid wastes may be safely disposed of to the sewers.

Wastes from Acetone-butanol Plants.—The slop from the acetone-butanol industry in which molasses is fermented may have a 5-day B.O.D. of 7,000 to 11,000 p.p.m.[1] It is thus a heavy waste. This waste may be digested anaerobically and stabilized by aerobic treatment. Some slop is concentrated, dried by the drum method, and

distributed to stock-feed mixers as a source of vitamin G, for such concentrates contain 70 to 100 micrograms of this vitamin per gram.

Wastes from Dairy Plants.—Very large quantities of dairy wastes are discarded each year to the sewer. The wastes include those from pasteurizing and bottling plants, from creameries, and from cheese factories. The total solid content of these wastes may vary from a fraction to about 4 per cent. Chemical precipitation of milk wastes is of little value in reducing the pollution load, since the lactose and other soluble solids that are not precipitated are readily attacked by bacteria and other microorganisms.

A very dilute milk waste will cause no trouble when disposed of along with other sewage. However, dairy wastes may frequently be the cause of considerable nuisance—the failure of sewage-treatment processes to operate successfully, the destruction of the normal life of a stream, etc.

Dairy wastes may be treated by one of the standard methods for treating domestic sewage or they may be fermented anaerobically according to the method developed by Boruff and Buswell,[1] wherein methane is produced as an important end product and is utilized for fuel purposes.

After certain preliminary treatment, which may involve the removal of settleable solids and grease, dairy wastes with a total solid content of no greater than 0.05 to 1 per cent[2] may be efficiently treated by one of the standard aerobic filter methods. The wastes may be passed through trickling filters with capacities for 500,000 to 2,000,000 gal. of liquid wastes per acre per day, through lath filters with capacities for 250,000 to 2,250,000 gal. per acre per day, or through sand filters, which may have capacities for 50,000 gal. per acre per day.[3]

Milk wastes may be disposed of by broad irrigation, but not infrequently disagreeable odors may arise from lagoons unless the wastes are prechlorinated.

In an anaerobic method recommended by Buswell and his associates,[1] 95 per cent, or greater, of the pollution load may be removed, while 8.3 to 12.4 cu. ft. of gas may be produced from each pound of dried solids added to the fermentation tanks. The filtered effluent from these tanks may be further treated, if desired, by one of the standard filter methods. The cost of this treatment, in which methane and carbon dioxide are the main gases produced, with small quantities of hydrogen and nitrogen, has been estimated by Buswell and his associates to be considerably less than that for present-day standard methods.

The anaerobic fermentation process developed by Boruff and Buswell may be carried out in a single tank or in two tanks connected in series. Before dairy wastes are introduced, the tank is filled about one-third full with well-digested sewage sludge and asbestos fibers and the remaining two-thirds with over-flow liquor (which has been permitted to settle) from an anaerobic sewage tank. The asbestos fibers serve as a rest or support for the bacteria after the sludge has been consumed. The sludge and overflow liquor supply the initial medium and the starter for the subsequent fermentations. The temperature is maintained at 27 to 29°C. The dairy wastes are fed into the tank slowly during its operation.

Organic matter is believed to be broken down to organic acids, such as propionic and acetic acids, by the anaerobic bacteria, which then convert these acids to methane and carbon dioxide, principally. If, during the fermentation, wastes are introduced too rapidly, organic acids may accumulate at the expense of gas production. An

[1] BORUFF, C. S., and A. M. BUSWELL, *Ind. Eng. Chem.*, **24**: 33 (1932).

[2] BUSWELL, A. M., C. S. BORUFF, and C. K. WIESMAN, *Ind. Eng. Chem.*, **24**: 1423 (1932).

undesirable flora may thus develop. Accordingly, during the first part of its operation, especial care must be exercised to favor the production of methane and carbon dioxide and a desirable bacterial flora in the tank. Should the contents of a tank become sour, the rate at which milk wastes are fed should be reduced or even stopped for a while. When two tanks are operated in series, liquor from the normal tank may be run into the contents of the tank that has become too acid. This practice usually restores a normal fermentation.

From 1.6 to 2.4 volumes of gas may be produced from each tank volume per day, with no "noticeable" quantity of sludge.

Wastes from Canneries.—Information concerning methods of treating cannery wastes may be obtained by studying *Bulletin* 28-L of the Research Laboratory of the National Canners Association.[1]

Additional Information.—The reader desiring detailed information concerning the biological, chemical, and engineering aspects of this subject may obtain it by studying some of the excellent texts and articles cited in the following list of references. Buswell and Hatfield's publication on "Anaerobic Fermentations" will be found of particular value. This publication contains a large number of references to the literature, including patent references, which are important in this as well as in other fields.

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